THE ROLE OF PRESYNAPTIC RECEPTORS IN THE CONTROL OF NEUROTRANSMITTER RELEASE

by

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Dedicated to Dad, Mum, Ranjna

and to the memory of Jyoti Patel.

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Statement in terms of Ph.D. regulation 2.4.15 of the postgraduate regulations of the University of Edinburgh

I declare that this thesis was composed by myself and all the experimental work described herein was performed by myself with the following exceptions:

The work published on the changes in glucose metabolism during convulsions in kindled rats (see Appendix II) was done in collaboration with Dr. D.H.R. Blackwood.

Chapter 2.1. Mr. D. Whale helped build the electrochemical detector.

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ABBREVIATIONS

ACh	-	Acetylcholine
Adr	-	Adrenaline
AMPT	-	α-Methyl-para-tyrosine
CSG	-	Cockroach salivary gland
DA	-	Dopamine
DOPAC	-	Dihydroxyphenylacetic acid
DOPEG	-	Dihydroxyphenyl glycol
EDTA	-	Ethyl-diamine tetra acetic acid
EGTA	-	[Ethylene-bis (oxyethylenenitride)] tetra acetic acid
GABA	-	γ-Amino butyric acid
^{3}H	-	Tritium
5-HIAA	-	5-Hydroxyindoleacetic acid
5-HT	-	5-Hydroxytryptamine
HVA	-	Homovanillic acid
KA	-	Kainic acid
L-DOPA	-	L-dihydroxyphenyl-alanine
MAO	-	Monoamine oxidase
MAOI	-	Monoamine oxidase inhibitor
ME	-	Median eminence
3-MT	-	3-Methoxytyramine
NA	-	Noradrenaline
nADA	-	N-acetyl-dopamine
6-OHDA	-	6-Hydroxydopamine
PBZ	-	Phenoxybenzamine
3PPP	-	3-(3-Hydroxyphenyl)-N-n-propylpiperidin hydrochloride
TH	-	Tyrosine hydroxylase

All other abbreviations are those in current scientific usage

ABSTRACT

A superfusion chamber was constructed to allow the monitoring of rapid changes in neurotransmitter overflow and metabolism from isolated tissue preparations *in vitro*, before, during and after application of depolarizing stimuli. An electrochemical detector (modified after Keller *et al.*, 1976; Kissinger *et al.*, 1973) was used in conjunction with high performance (ion-pair) chromatography to allow the monitoring of endogenous catecholaminergic transmitters and their metabolites in the superfusate from striatal slices.

High K^+ ion concentration and electrical stimulation, Ca^{++} dependently, evoked the overflow of DA and DOPAC. While the small electrically evoked overflow of DA was greatly enhanced by uptake inhibitors, the already larger DA overflow with high K^+ stimulation remained unaffected, suggesting that uptake processes are inoperative during high K^+ stimulation. The high basal and evoked overflow of DOPAC remained unaltered under conditions of uptake inhibition, implying a presynaptic site of origin of DOPAC. Changes in DOPAC overflow appeared to follow changes in DA synthesis better than changes in DA release.

Muscarinic agonists enhanced the overflow of both DA and DOPAC evoked by high K^+ or electrical stimulation even in the presence of uptake inhibitors implying that the release and turnover of DA were enhanced. The simultaneous determination of ^3H (^3H-DA preincubation) and endogenous DA/DOPAC overflow suggested that although the uptake of DA was partially inhibited, ^3H overflow followed changes in DA overflow only under control conditions. When the rate of synthesis and release was altered by drugs (uptake inhibitors, muscarinic agonists, dopaminergic agents), ^3H overflow failed to follow accurately changes in DA overflow, adding weight to the suggestion of preferential release of newly-synthesized DA. This hypothesis was further supported by examining the effects of DA synthesis inhibition on DA overflow.

Dopaminergic agonists (3PPP) prevented the increase of DA/DOPAC basal overflow found in the absence of depolarizing stimuli *in vitro* as has been reported *in vivo* after impulse inhibition. 3PPP also appeared to decrease synthesis and release mainly of newly synthesized DA, hence simultaneous ^3H overflow was slightly but not significantly altered under these conditions. Haloperidol appeared to increase the synthesis and release of DA, again the effect of haloperidol was most marked on endogenous rather than preincubated ^3H-DA overflow.

The release of endogenous DA from the median eminence and retina of rats and from the cockroach salivary gland is also described.

CHAPTER I

Introduction

1.1 Historical Introduction

The presence of presynaptic receptors on nerve terminals was suggested in the early part of this century, by the observation that Acetyl Choline (ACh) and cholinesterase inhibitors (eserine and prostigmin) initiated depolarizations of motor nerve terminals, which would propagate antidromically (Masland and Wigton, 1940), as well as causing muscular twitching (Langley and Kato, 1915). More detailed studies by Feng and Li (1941) and Eccles *et al.* (1942) showed that while repetitive firing of the nerve endings can arise from various changes in the chemical environment, the effects produced by cholinesterase inhibitors are almost certainly due to the local accumulation of ACh around the axon terminal after each impulse. However, these cholinergic (nicotinic) presynaptic receptors are unusual in that they can generate antidromic action potentials, as well as inducing release of transmitter, not only from motor nerve endings but also from postganglionic noradrenergic nerve fibres (Hoffman *et al.*, 1945; Ferry, 1963).

In retrospect, initial evidence for the existence of stimulation induced release regulating presynaptic receptors came from the work of Brown and Gillespie (1957). Working on the perfused cat spleen, the authors noted that the overflow of NA per impulse, into the venous blood was related to the frequency of stimulation of the splenic nerve, being maximal by about 30Hz. The overflow was unaltered by inhibitors of monoamineoxidase or catechol-O-methyl transferase but greatly increased by α-adrenergic antagonists (phenoxybenzamine, PBZ, and dibenamine), i.e. by as much as 10-fold at 10Hz with smaller increases seen at 30Hz stimulation. At the time the authors concluded that NA once released first bound to the postsynaptic receptors and was sub-

sequently taken up and metabolized, but when these receptors were blocked, the released transmitter escaped into the overflow immediately.

The action of α-adrenergic antagonists on NA overflow generated much interest and controversy as to their mechanism of action. Paton (1960), working on the overflow of NA and Adr from the Adrenal medulla, suggested that the uptake referred to by Brown and Gillespie (1957) and Brown (1960) may be intra- rather than extra-neuronal. Although at the time Brown (1960) considered this "a most attractive heresy", the whole concept of neuronal uptake was quickly established during the early 1960's, especially after the availability of radiolabelled catecholamines with a high specific activity (for a complete discussion of the evidence see Iversen, 1965, 1967, 1975). With increasing evidence for neuronal and extraneuronal uptake the important distinction between *release* (total amount of transmitter released by nerve terminals), and *overflow* (diffusion of part of the released transmitter into the perfusing/incubation fluid) was made (see Figure 1.1). The common suggestion in both hypothesis presented above, therefore, was that α-antagonists induced an increase of NA overflow but not of NA release.

Thoenen *et al.* (1964) provided some evidence in favour of Paton's (1960) hypothesis by showing that the "uptake inhibition" of NA and the inhibition of splenic contractions during stimulation of the nerve had a different time-course and sensitivity to PBZ, suggesting dual sites of action. Although other workers (Hertting *et al.*, 1961; Iversen, 1965; Iversen and Langer, 1969) were able to show inhibition of neuronal uptake by PBZ several pieces of data did not fit into this explanation. Most importantly, some potent inhibitors of NA uptake (e.g. cocaine and desmethylimipramine) did not markedly increase NA overflow, and PBZ and other α-adrenergic antagonists, were effective

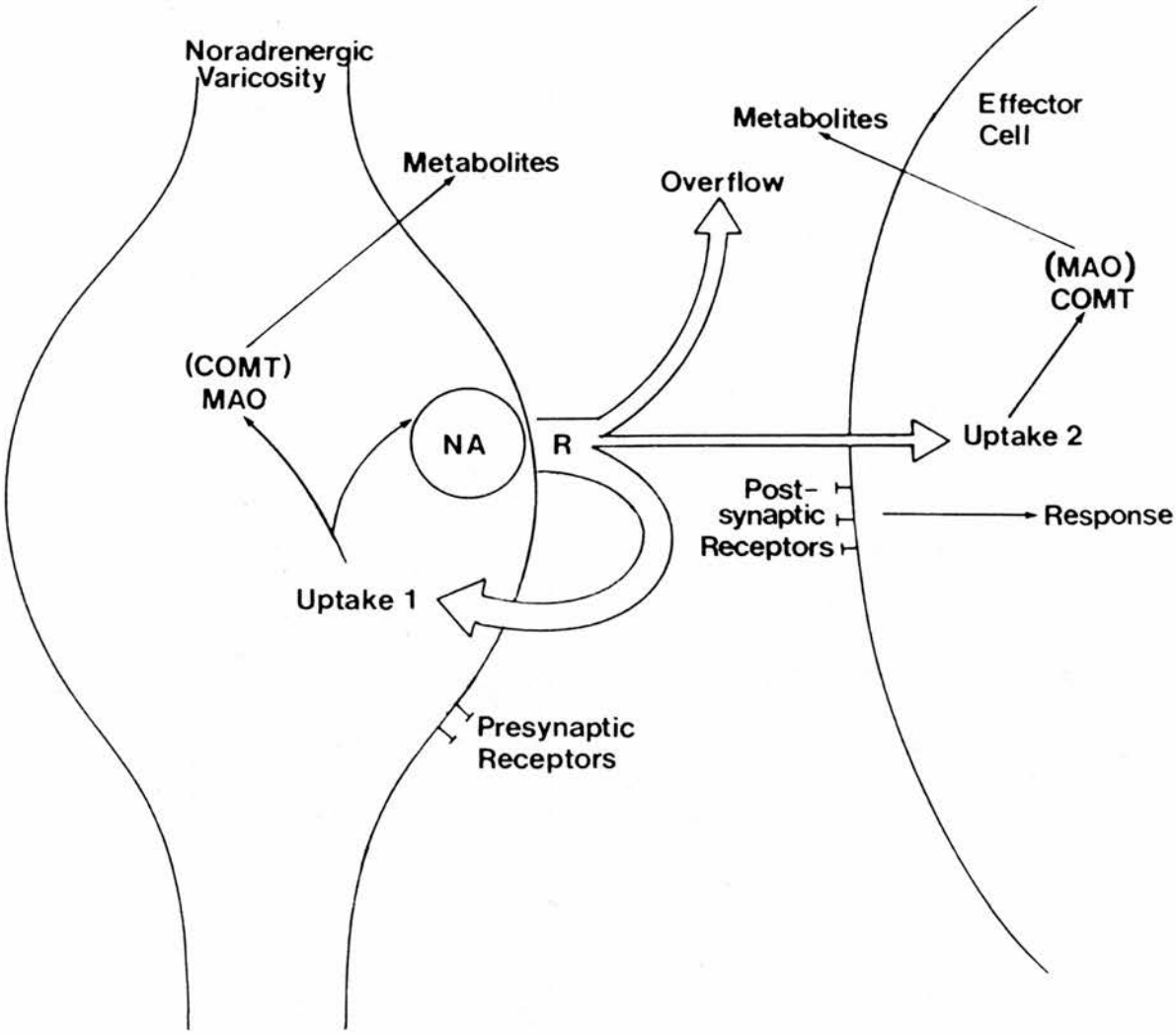


FIGURE 1.1: Main sites of loss of noradrenaline (NA) released during sympathetic nerve stimulation.

R - Release; total amount of NA release by the nerve terminal during nerve stimulation.

Uptake 1 - neuronal uptake, the NA being subsequently recycled or deaminated.

Uptake 2 - extra neuronal uptake and subsequent catabolism, mainly by 'o'-methylation.

Overflow - Fraction of released NA diffusing into the perfusing/ superfusing medium.

MAO - monoamine oxidase.

COMT - catechol-O-methyl transferase.

in increasing NA overflow even in the presence of these uptake inhibitors (Blakeley *et al.*, 1963; Geffen, 1965; Kirpekar and Misu, 1967). Subsequently, Langer (1970) and Langer and Vogt (1971) showed that although PBZ was able to inhibit intra- and extra-neuronal uptake and so inhibit the degradation of NA, this effect was not important in the action of α-blockers on NA overflow, as indicated by the lack of effect of phentolamine on NA metabolism but its effectiveness on increasing NA overflow.

Around that time, Davis *et al.* (1968) reported the release of prostaglandins (PGE_2) along with NA, into splenic venous blood during splenic nerve stimulation. With the finding that PG's of the E series could reduce NA overflow during nerve stimulations (Hedqvist, 1969a) and that PGE could antagonize the effects of PBZ on this system (Hedqvist, 1969b) came the "transsynaptic regulation of transmitter release" hypothesis (Hedqvist, 1969a,b; Haggendal, 1969). It was suggested by these authors that the release of NA (i.e. changes in overflow reflected changes in the release not uptake of NA) by some transsynaptic signal (e.g. PG's, changes in intrasynaptic ion concentrations leading to changes in membrane potential or the reduced availability of Ca^{++} ions for stimulation-secretion coupling) which was inversely related to the intensity of effector cell activity (although "presynaptic" actions were not ruled out). However, this hypothesis had to be abandoned with the finding that α-agonists and antagonists could modify the overflow of NA in tissues where the postsynaptic response was mediated by β-receptors (e.g. heart) where overflow could be shown to directly relate to effector cell response (Kaumann, 1970; Starke, 1971).

The finding that PBZ is effective in doses lower than those required for uptake inhibition and in tissues where the postsynaptic

effects are mediated by β-receptors (Starke *et al.*, 1971) effectively ended the ideas that uptake (neuronal and extraneuronal) or transsynaptic regulation were major mechanisms affected by α-adrenergic antagonists.

Subsequently it was shown that α-adrenergic agonists could depress the stimulation evoked overflow of NA from the cat's spleen (Starke, 1971). So, in 1971 several authors working independently in different laboratories, more or less simultaneously put forward the "presynaptic-α-receptor mediated feedback hypothesis" in the regulation of stimulation induced NA release (Farnebo and Hamberger, 1971a; Kirpekar and Puig, 1971; Langer *et al.*, 1971; Starke, 1971). Starke (1971) proposed that:

> "the adrenergic nerve terminals are endowed with structures related to the α-adrenoceptive sites of effector cells. On reaction with α-stimulants, e.g. with liberated NA, these neuronal α-receptors mediate ... inhibition of NA liberation; in the presence of α-blockers, this restriction is attenuated".

Earlier studies had demonstrated the existence of other types of presynaptic receptor systems, e.g. inhibitory α-presynaptic receptors on parasympathetic nerves (Paton and Vizi, 1969) and on motor nerves (Kr njevic and Miledi, 1958; Bowman and Nott, 1969); and inhibitory muscarinic presynaptic receptors on noradrenergic nerves (Lindmar *et al.*, 1968), regulating the release of the respective transmitters.

Studies on the overflow of false transmitters (see Muscholl, 1972) lent further support to the hypothesis that the release of neurotransmitters was regulated by presynaptic receptors. Muscholl (1973) demonstrated that the evoked overflow of α-methyladrenaline from the rabbit heart, could be reduced by the muscarinic agonist, methacoline, as was the overflow of endogenous NA.

The discovery by Frank and Fourtes (1957) that depolarizations induced in gastrocnemius motor neurons on stimulation of gastrocnemius nerves, could be reduced by a preceding (7 msec) volley in the hamstring afferents of the cat, without itself inducing hyperpolarization (or changes in excitability), led to the discovery of a different form of "presynaptic inhibition". Part of the difference stems from the fact that the evidence for this type of presynaptic inhibition is almost exclusively electrophysiological and morphological, rather than neurochemical, and hence it has developed independently of the rest of the evidence for presynaptic inhibition stated here (see Schmidt, 1971; Ryall, 1978, for reviews).

1.2 Nature of the presynaptic receptor mediated feedback control of neurotransmitter release in the peripheral nervous system

The suggestion that the release of NA is regulated by presynaptic α-adrenoceptors generated a lot of interest. In the eleven years that have followed, an enormous amount of evidence has been gathered to support the ideas that various types of presynaptic receptors can modulate the release of NA from the peripheral nervous system (see page 7 and 9). Majority of the papers concerning the release of NA use the overflow of ^{3}H (after preincubation of the tissue with ^{3}H-NA) as an index of endogenous NA release. The basal overflow of ^{3}H from tissues precincubated with ^{3}H-NA, however, consists mainly of the ^{3}H-metabolites of the ^{3}H-NA (Jayasundar and Vohra, 1978), and even during stimulation only 25-45% of the overflow of ^{3}H can be attributed to authentic ^{3}H-NA (Langer and Enero, 1974; Brandao *et al.*, 1980; Graefe *et al.*, 1973). Most authors refer to the overflow of ^{3}H as an overflow of ^{3}H-NA, complications may arise from this, as this measure

is probably a better index of NA release and metabolism than of NA release alone.

In addition to these difficulties, with improved methods of separation and detection of NA and its metabolites, it is becoming clear that the metabolism of endogenous and exogenous NA is different (Graefe, 1981; Starke *et al.*, 1981; Majewski *et al.*, 1982).

After all the possibilities of drug induced effects on the sites of loss of neurotransmitters (see Figure 1.1) have been ruled out, there is evidence to support the claim that the following presynaptic receptors modulate the induced release of NA from the peripheral nervous system:

1. Facilitatory:
   a) β-Adrenergic (Weinstock *et al.*, 1978; Dahlof *et al.*, 1980; Dahlof, 1981).
   b) Angiotensin II (Zimmerman and Kraft, 1979; Gothert, 1977).
   c) Nicotinic (Nedergaard and Schrold, 1977; Kirpekar *et al.*, 1980).
2. Inhibitory
   a) α-Adrenergic (Gothert, 1977; Alberts *et al.*, 1981).
   b) Prostaglandin (Hedqvist, 1977; Stjarne, 1979).
   c) Dopamine (Hope *et al.*, 1979; Fuder and Muscholl, 1978; Dubocovich and Langer, 1980).
   d) Muscarinic (Gothert, 1977; Muscholl *et al.*, 1979).
   e) Opiates (Henderson *et al.*, 1979).
   f) Adenosine (Khan and Malik, 1980; Clanachan, 1979).

Evidence for GABA (Weitzell and Starke, 1980), Serotonin (Martinez and Lokhandwala, 1980), and Histamine (Powell, 1979), presynaptic receptors is as yet incomplete.

Similarly, a whole range of ACh release modulating presynaptic receptors have been reported, both on motor and pre-post-ganglionic cholinergic neurones (see Vizi, 1979; Miyamoto, 1978; Starke, 1977, 1981b; Gustafsson, 1980; Fredholm and Hedqvist, 1980).

Since the subject has been extensively reviewed by numerous authors (Stjarne, 1975b; Baldessarini, 1975; Starke, 1977, 1981b; Westfall, 1977; Vizi, 1979; Langer, 1974, 1977, 1979, 1980, 1981; Rand *et al.*, 1980; Gillespie, 1980), only some of the more important and controversial areas will be considered here.

1. Tissue and species differences

Numerous presynaptic receptor mediated controls of NA overflow have been described (see Figure 1.2 and above), from a variety of tissue preparations. However, not all the presynaptic receptors can be demonstrated in every tissue preparation. Angiotensin, for example, increases the evoked overflow of NA from the rat vas deferens (Johnson *et al.*, 1974) and the rabbit vas deferens (Henderson and Hughes, 1974) but not the mouse vas deferens (Henderson and Hughes, 1974). Such differences occur intraspecifically as well, e.g. PG's are effective in decreasing NA overflow in the cat spleen (Hedqvist *et al.*, 1971) but they have no such effect in the cat nictitating membrane (Langer *et al.*, 1975a). In a careful survey of the presynaptic receptors on the rabbit pulmonary artery, Endo *et al.*, (1977) found release modulating α-adrenergic, angiotensin, muscarinic, nicotinic and PG type receptors, however, there appeared to be no β-adrenergic or DA presynaptic receptors. Although the complete absence of presynaptic receptors is difficult to establish, the significance of tissue and species difference in the effectiveness of presynaptic receptor systems is obscure.

Interestingly, noradrenergic neurons can take up and release adrenaline (as a false transmitter), after which presynaptic β-receptors which are not normally functional, become active (Guimaraes *et al.*, 1978; Rand *et al.*, 1979). Similarly, when noradrenergic nerves are made to release DA (by preincubation with ^{3}H-DA and a dopamine -

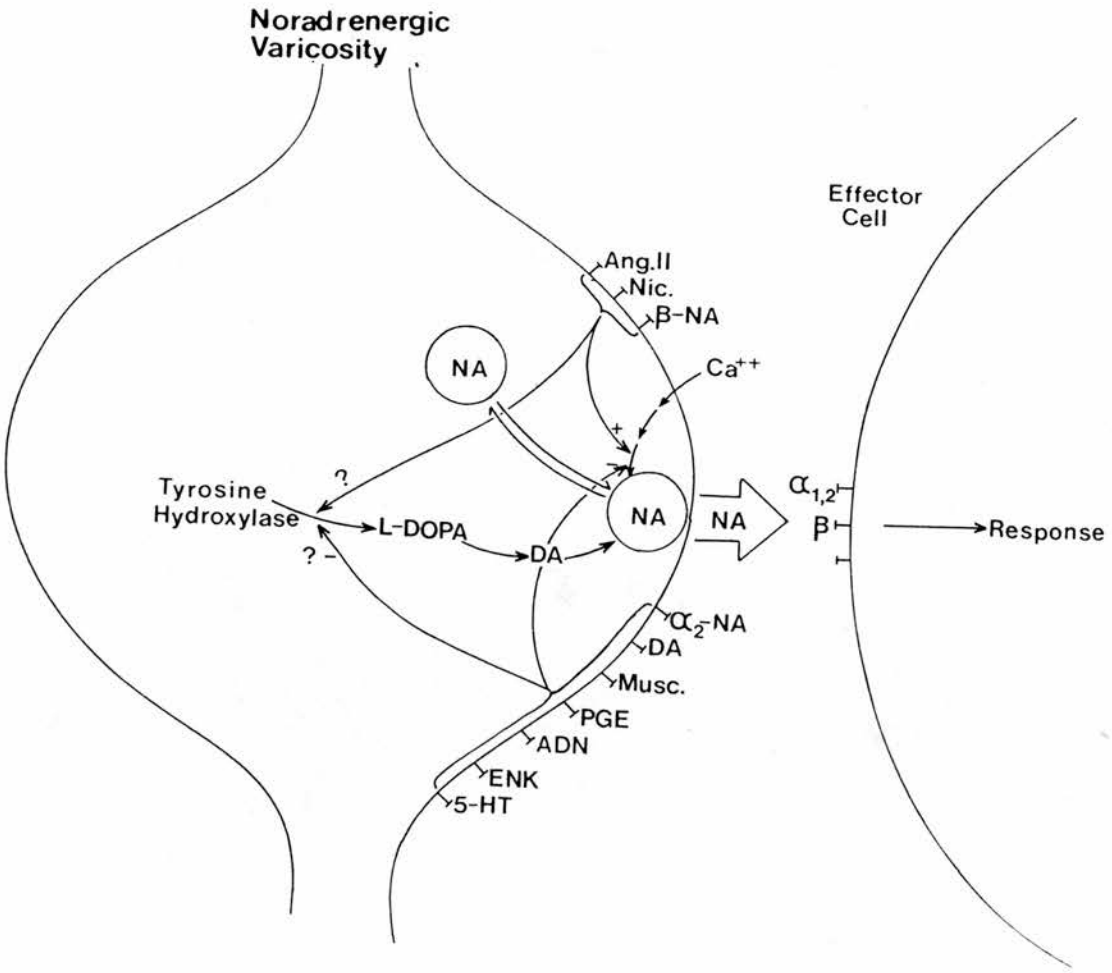


FIGURE 1.2: Schematic representation of the pre- and post-synaptic receptors in a noradrenergic neuroeffector junction in the peripheral nervous system.

Presynaptic α_2-NA, dopamine (DA), muscarinic (Musc) cholinergic, prostaglandin E (PGE), adenosine (ADN), enkephalin (ENK) opiate, and serotonergic (5-HT) receptors mediate inhibition of the evoked release of NA.

Presynaptic angiotensin II (Ang.II), nicotinic (Nic) cholinergic and β-noradrenergic (β-NA) receptors mediate facilitation of evoked NA release.

The effect of presynaptic receptors on NA synthesis (tyrosine hydroxylase activity) is incomplete.

The post-synaptic response may be mediated by α_1, α_2 or β-noradrenergic and other receptors.

β-hydroxylase (D-β-H) inhibitor [Hope *et al.*, 1979]; or by administering L-DOPA [Lokhandwala and Buckley, 1978]), presynaptic DA receptors which are normally 'silent' become active in their ability to depress the induced release of the transmitter. These findings suggest that some presynaptic receptors may be silent under normal physiological conditions.

2. The effect of presynaptic receptor mechanisms on the release of newly synthesised NA

The release of both endogenous NA (Bell and Vogt, 1971; Geffen, 1965) and previously taken up ^{3}H-NA (Stjarne, 1974; Starke, 1972; Langer, 1970) is enhanced by α-antagonists, despite differences in their presynaptic storage and metabolism (Brandao *et al.*, 1980; Starke *et al.*, 1981; Majewski *et al.*, 1982) indicating that the effects of presynaptic receptors are at least partially independent of de novo synthesis.

There is some evidence to suggest that newly synthesised NA is preferentially released (Stjarne and Wennmalm, 1970; Kopin *et al.*, 1968; Wennmalm, 1971). Despite findings that α-antagonists can increase synthesis of NA (Dairman *et al.*, 1968; Ikeno *et al.*, 1981; Dairman and Udenfriend, 1970) crucial evidence for the influence (or the lack of it) of presynaptic receptor mechanisms on the release of newly synthesised NA appears to be missing from the literature. Such evidence may help resolve some of the differences of opinion that exist in the current literature.

3. One pulse release of NA

Alpha-antagonists do not appear to modify the overflow of ^{3}H-NA in response to one electrical pulse, but are effective after two or more successive pulses (Rand *et al.*, 1973; Markiewicz *et al.*, 1980; Wakade

and Wakade, 1981), as would be expected from the feedback hypothesis, since no prior released NA is available to stimulate presynaptic α-receptors during a one pulse stimulation.

The negative results reported by Kalsner (1979) and Angus and Korner (1980), both using PBZ, may be explained by the uptake inhibition effects of PBZ when used in high doses (c.f. Story *et al.*, 1981).

Exogenous NA reduces the overflow of ^{3}H-NA (in the presence of uptake inhibitors to reduce the effects of uptake of "cold" NA diluting the ^{3}H-NA stores), during one pulse or trains of pulses, an effect that is stereospecific (Rand *et al.*, 1980; Starke, 1972b; Stjarne, 1974). Endogenously released NA reduces the effectiveness of α-blockers at the presynaptic site, especially in the presence of uptake inhibitors (Reichenbacher *et al.*, 1982; Hope *et al.*, 1976; Dubocovich *et al.*, 1979; for more detailed analysis see Baumann and Koella, 1980; Pelayo *et al.*, 1980). Depletion of NA stores with reserpine or α-methyl-para-tyrosine (AMPT) pretreatment reduces the effectiveness of α-adrenergic antagonists on increasing the stimulation induced overflow of ^{3}H-NA (Cubeddu and Weiner, 1975; Enero and Langer, 1973); because the biophase concentration of NA available to stimulate presynaptic α-adrenoceptors is lowered.

4. Frequency dependence

The inhibition of nerve stimulated release of NA by α-agonists is inversely correlated with the frequency of nerve stimulation (Starke *et al.*, 1975; Westfall and Leighton, 1976; Langer *et al.*, 1975a). This has been interpreted as follows: at high frequencies of stimulation (or with uptake blockers) NA levels in the synaptic cleft will be elevated to the point where presynaptic α-receptors are maximally

activated (by endogenous NA) hence addition of α-agonists will show no effect.

Alberts *et al.* (1981) have suggested that at high frequencies of stimulation, the decreased "Ca^{++} availability" induced by presynaptic α-receptors is ineffective as there is sufficient intracellular Ca^{++} to overcome this inhibition. So, presynaptic α-adrenergic inhibition of release and "facilitation" of release (seen at high frequency stimulation) are seen as being mutually antagonistic.

5. Regulation of Ca^{++} dependent (exocytotic) release

During nerve stimulation the release of NA is accompanied by the release of other contents of "noradrenergic vesicles", i.e. D-β-H, chromogranins, ATP (Geffen and Livett, 1971; De Potter *et al.*, 1972); findings that are taken as evidence for "exocytotic release". The finding that increases in the stimulation evoked overflow of NA produced by α-adrenoceptor blocking drugs is accompanied by an increase in release of D-β-H (De Potter *et al.*, 1972; Johnson *et al.*, 1971; Cubeddu *et al.*, 1974a,b; Cubeddu and Weiner, 1975; see, however, Smith *et al.*, 1970) suggested that the increased overflow of NA was due to an increased "exocytotic" release of NA. Uptake inhibitors slightly decrease the release of D-β-H, while increasing the stimulation evoked overflow of ^{3}H-NA (De Potter *et al.*, 1971), consistent with the idea that the increased biophase concentration of NA due to the uptake inhibitors, had led to a feedback inhibition of actual NA release, reflected by the decreased D-β-H release.

The finding that the tyramine induced overflow of NA (non-exocytotic, Ca^{++} independent release; Chubb *et al.*, 1972) was unaltered by α-antagonists (Starke and Montel, 1974), suggesting that only Ca^{++} dependent release processes are regulated by presynaptic receptors.

The *basal overflow* (overflow of transmitters/metabolites in the absence of depolarizing stimuli), of catecholamines is also Ca^{++} independent (Henseling *et al.*, 1976) and remains unaffected by α-adrenergic antagonists. The potassium induced release of NA is Ca^{++} dependent and is influenced by α-adrenergic agents (Starke and Montel, 1974; Stjarne, 1973b; Alberts *et al.*, 1981) as is the release induced by nicotinic agents (Furchgott *et al.*, 1975).

6. Topographic location of presynaptic receptors

Direct evidence for a presynaptic location of release modulating receptors is lacking, there are however several pieces of evidence suggesting that "presynaptic" receptors are located somewhere on nerve terminals.

Noradrenaline release modulating α- and β-adrenoceptors have been demonstrated on the axonal sprouts of sympathetic ganglion cells grown in organ culture (Vogel *et al.*, 1972; Weinstock *et al.*, 1978) where no effector cells are present.

Increases in the stimulation induced overflow of NA by α-antagonists are unaffected by the atrophy of postsynaptic effector cells, as demonstrated by Filinger *et al.* (1978), after duct ligation of the rat submaxillary gland, which results in the atrophy of gland cells but not nerve terminals.

After degeneration of noradrenergic nerve endings in the rat heart by chemical sympathectomy with 6-hydroxydopamine (6-OHDA), there is a significant reduction in the specific binding of an α-adrenoceptor ligand (^{3}H-dihydroergocryptine; Story *et al.*, 1979). Earlier, Sharma and Banerjee (1977) reported the reduction of muscarinic binding sites (^{3}H-quinuclidinyl benzilate) in the rat heart after 6-OHDA treatment; Story *et al.* (1979a,b), however, were unable to repeat this and

suggested that muscarinic receptors were located largely on effector cells. Recently, Dahlof (1981) demonstrated a decreased binding of a β-adrenergic ligand ([^{125}I]-iodo-hydroxybenzylpindolol) in the cat spleen, but not the heart, after 6-OHDA treatment and suggested that in the cat spleen, β-adrenoceptors were located mainly on sympathetic nerve terminals. Small decreases in muscarinic antagonist binding in the striatum, ipsilateral to a unilateral 6-OHDA lesion may be due to loss of presynaptic receptors or a decrease in soma-dendritic receptors (due, for example, to an ovaractivity of effector cells after the removal of an inhibitory influence; Kato *et al.*, 1978; Giorgi and Rubio, 1981). Similar studies involving lesions of primary afferents to specific brain areas, in an attempt to find decreases in the binding of specific ligands after degeneration of nerve terminals, have led to conflicting reports (see Starke, 1981b), reaffirming the view that binding studies should be viewed with caution.

Drug induced modulation of the overflow of neurotransmitters from brain synaptosomal preparations (Wemer *et al.*, 1981; De Langen *et al.*, 1979; De Belleroche and Bradford, 1978), and presynaptic modulation of the release induced local depolarization of terminals (e.g. with high K^+ ion concentrations; Giorguieff-Chesselet *et al.*, 1979; Starke and Montel, 1974; Wemer *et al.*, 1981), from isolated preparations (especially where no cell bodies are present), all add further weight to the suggestion of a presynaptic topographic location of presynaptic receptors.

An "extra-synaptic" location of presynaptic receptors has been suggested based on differences in the potency of antagonists to endogenously released and exogenously applied NA at the presynaptic receptor sites (Langer *et al.*, 1981a,b; Wilffert, 1981). The effect of drugs on the release of NA from ligated nerves (Esquerro *et al.*, 1980a,b) may provide some interesting results.

7. Pharmacological difference between pre- and post-synaptic receptors

In retrospect, the earliest clue to a difference between pre- and post-synaptic receptors came from the work of Thoenen *et al.* (1964) (see 1.1). Langer (1973), comparing the pre- and post-synaptic effects of NA and DA in the cat spleen, suggested that the pre- and post-synaptic α-adrenoceptors, were probably different. This was supported by Cubeddu *et al.*,(1974c) and Dubocovich and Langer (1974), when they showed that PBZ, in the same preparation, was 30-100 times more potent in blocking post- than pre-synaptic receptor mediated effects. Starke *et al.*, (1975a,b) compared the relative potencies of several α-adrenergic agonists on pre- and post-synaptic α-receptors in the rabbit pulmonary artery. They reported that methoxamine and phenylephrine had a preferential post-synaptic action, i.e. contraction of the preparation. The actions of oxymetazoline, α-methylnoradrenaline, tramazoline and clonidine on the other hand were preferentially pre-synaptic, i.e. inhibition of transmitter release. Noradrenaline, adrenaline and naphazoline were equally effective at both sites. Drew (1977, 1978) extended these studies in the rat vas deferens and guinea-pig ileum.

Langer (1974) originally suggested the terms α_1 for the post-synaptic α-adrenoceptor, and α_2 for the pre-synaptic α-adrenoceptor. Although many authors have since demonstrated a pharmacological difference between pre- and post-synaptic receptors (Wikberg, 1979; Starke, 1981a), binding studies with ^{3}H-dihydroergocryptine (DHE), and its displacement by preferential pre- or post-synaptic antagonists (Hoffman *et al.*, 1979), demonstrated that α_2-adrenoceptors may also be located post-synaptically on effector cells, functionally this has also found to be so (Docherty and Starke, 1982). The subclassification

of α-adrenoceptors as α_1 and α_2 should therefore be based only on pharmacological characteristics.

Comparisons of the pharmacological properties of pre- and post-synaptic muscarinic receptors suggest that the two are identical in the heart (Fuder *et al.*, 1981a,b) and neocortex (Bowen and Marek, 1982).

8. Mechanism of action

Relatively little is known about the mechanism of action of pre-synaptic receptor mediated actions.

The observations that presynaptic receptors: 1) modify only Ca^{++} dependent release processes; 2) action is inversely related to the frequency of stimulation; 3) are most active in low Ca^{++} media and ineffective when the extracellular Ca^{++} ion concentration is high (Stjarne, 1975b; Langer *et al.*, 1975a; Westfall and Leighton, 1976; Drew, 1978a); have led to the suggestion that presynaptic receptors modify "Ca^{++} availability" in the presynaptic terminal (Starke, 1977; Westfall, 1977; Langer, 1977, 1979; Gillespie, 1980; Rand *et al.*, 1980; see however Kalsner, 1981).

Stjarne (1978) and his colleagues (Alberts *et al.*, 1981) have suggested that control of invasion (recruitment) of varicosities by action potentials is perhaps more important than the regulation of the excitation-secretion coupling (both involving utilization of external Ca^{++} ions) in the mode of action of presynaptic receptors, however direct evidence is lacking. Similarly, the proposal by Vizi (1978, 1979; see however Vizi and Vyskocil, 1979) of the involvement of ATPases lacks direct evidence (for evidence against, see Alberts *et al.*, 1981; Wakade, 1981).

Although cyclic AMP (Phosphodiesterase inhibitors and stable analogues of cAMP) does increase the overflow of ^{3}H-NA (Cubeddu

et al., 1974b,1975; Stjarne, 1976; North and Vitek, 1980) no correlation was found between this and the presynaptic effects of α-antagonists (see, however, Stjarne *et al.*, 1979), opiates and PGE_2. Although Stjarne *et al.* (1979) report no effect of cGMP in the guinea-pig vas deferens, there is fairly convincing evidence that the presynaptic α-adrenoceptor effects on NA overflow from the pineal gland, may be mediated by cGMP (O'Dea and Zatz, 1976; Pelayo *et al.*, 1978). Yonehara *et al.* (1980) have provided some evidence that cGMP may be involved in the presynaptic muscarinic inhibition of ^{3}H-ACh overflow from rat cortex. Clearly, much more information is required to sort out the role of cyclic nucleotides in the control of neurotransmitter release.

1.3 Dopaminergic presynaptic receptors in the central nervous system

1.3.1:

Strong evidence for a presynaptic receptor mediated regulation of the release of neurotransmitters in the CNS began to emerge in the early 1970's (Farnebo and Hamberger, 1971b). Although in the periphery a direct action of presynaptic modulators on nerve endings has come to be generally accepted, such an action in the CNS is less certain.

Within the CNS, as cells may have several receptor types/subtypes regulating both the generation of action potentials (soma-dendritic or postsynaptic receptors) and presynaptic effects, the term 'presynaptic receptor' therefore, is used cautiously for sites that are: 1) distinct from soma-dendritic receptors and do not control the generation of action potentials in the perikarya; 2) modify Ca^{++} dependent release processes and/or neurotransmitter 'turnover'; 3) are not necessarily located exclusively within synapses. The term 'autoreceptor' (as suggested by Carlsson, 1975, to emphasise a neuron's sensitivity to its own transmitter, rather than its functional/locational distinction) will be avoided in this text, as it has not been generally accepted (except perhaps for dopaminergic 'autoreceptors') and is more ambigious (Starke and Langer, 1979).

In order to avoid some of the variables, brain slice preparations have been used with some advantage in the field of transmitter release studies. They allow careful control of the chemical environment, and avoid the loss of metabolites into the blood stream. Furthermore, drug induced alterations in the firing rate of cells can be avoided by using slices which do not have cell bodies.

Rat striatal slices made from the head of the caudate, for example, contain the terminals of the dopaminergic nigro-striatal pathway, the cell bodies being in the A9 region (Ungerstedt, 1971). Another advantage of this preparation is that while DA is present in very high concentrations, there are little or no other catecholamines, and only very small amounts of indoleamines (Fonnum and Walaas, 1979). Dissociation from other DA rich areas is also important as uptake, release and metabolism of DA (and their controls) appear to differ in the different areas of the brain (Broxterman *et al.*, 1981; Annunziato, 1979; Annunziato *et al.*, 1980; Nicolaou, 1980). Hence much of the evidence for presynaptic receptor control of DA release and metabolism comes from studies on the striatum and in particular from striatal slices. Farnebo (1971) has, however, pointed out that striatal slices undergo changes in their microscopic appearance during incubation. Synaptosomal preparations can also be induced to release neurotransmitters (Ca^{++} dependently) while avoiding any short interneuron network (Jones, 1975), presently available synaptosomal preparations, however, are heterogenous.

With very few exceptions, studies on the presynaptic regulation of transmitter release have relied on the use of labelled transmitters, after preincubation with the labelled transmitter or its precursor. There are several possible sources of error in this technique which cast some doubt on the validity of using ^{3}H (or ^{14}C) overflow as an index of endogenous transmitter release.

1. Labelled compounds may be taken up into cells other than those containing the endogenous transmitter, and may subsequently be released from the "wrong" structure. For example, ^{3}H-DA can be taken up and be converted to ^{3}H-NA, as well as being released

together with NA as a "false transmitter" from noradrenergic nerve endings (Farnebo *et al.*, 1971). Similarly, ^{3}H-NA may be released from dopaminergic nerve endings or adrenaline containing neurons, or the recently discovered "amine handling cells" (Howe *et al.*, 1980).

Although such difficulties may be reduced by choosing appropriate brain areas, e.g. the striatum for studying ^{3}H-DA release, the occipital cortex for ^{3}H-NA release, the release of label from other structures, e.g. glia (Jafe and Cuello, 1981) or blood vessels, remains in most cases to be investigated.

2. Majority of the papers published on the overflow of ^{3}H transmitters (previously preincubated), use the overflow of total ^{3}H as an index of authentic transmitter release. As pointed out earlier (see page 6), the major part of the basal overflow of ^{3}H after preloading with ^{3}H-NA or ^{3}H-DA appears to be associated with ^{3}H-DOPEG and ^{3}H-DOPAC respectively (Farah *et al.*, 1977; Taube *et al.*, 1977). In the case of ^{3}H-DA, basal overflow consists of 61-68% ^{3}H-DOPAC and only 6-10% ^{3}H-DA (Cubeddu *et al.*, 1979a,b; Zumstein *et al.*, 1981). Westfall *et al.* (1976b) reported much lower ^{3}H-DOPAC levels possibly because the very low recovery of ^{3}H-DOPAC by their technique (58%) was not corrected for. The work of these authors has emphasised the dangers of false interpretation of results when the chemical nature of the released label is not known.

To try and circumvent the above mentioned problems, many authors have resorted to the use of monoamineoxidase inhibitors (MAOI). However, when pargyline is present during the preincubation with ^{3}H-DA, there appears to be a large increase in the accumula-

tion of ^{3}H by the tissues, and in the K^{+} (20mM) and amphetamine induced overflow of the ^{3}H (Arbilla and Langer, 1980), suggesting that MAOI alter the intraneuronal distribution of ^{3}H-DA, which in turn alters release. Pargyline present only during superfusion of striatal tissue preloaded with ^{3}H-DA reduces the basal and evoked overflow of ^{3}H (Zumstein *et al.*, 1981). Subsequent to a rise in DA concentration after MAOI (Glowinski *et al.*, 1972; Kehr, 1976), there is a decrease in the synthesis rate of DA (Roth *et al.*, 1973; Javoy *et al.*, 1973) which probably also affects release.

3. Intraneuronal compartmentation of dopamine: Van Rossum *et al.* (1962) and Weissman and Koe (1965) showed that while the behavioural effects of amphetamine persisted even after depletion of catecholamines with reserpine, they were quickly abolished by AMPT even before the severe depletion of catecholamines, suggesting that the effects of amphetamine were due to the release of newly synthesised catecholamines. Subsequent work by Enna *et al.*, (1973) and Chieuh and Moore (1974) on the *in vivo* release of labelled catecholamines suggested that although amphetamine initially released both stored and newly synthesised catecholamines, maintenance of the amphetamine induced release depended on *de novo* synthesis. Initial studies on the disappearance of DA after synthesis inhibition (AMPT) showed a log-linear decline of DA for several hours (Neff and Costa, 1968), but subsequent detailed work showed an initial fast phase followed by a slower decline phase (Javoy and Glowinski, 1971). The authors suggested that the two phases represented a small (26%) "newly-synthesised releasable pool" and a "storage pool" of DA. Doteuchi *et al.*, (1974), however, have suggested that the result may be an artifact caused by metabolites of AMPT, and if

present the releasable newly-synthesised pool cannot be more than 5% of the total DA in the neurons. Since then, work by Glowinski, Besson, Cheramy, Javoy and Thierry on the release of ^{3}H-DA, recently synthesised from ^{3}H-tyrosine, both *in vivo* and *in vitro* has suggested that there is preferential release of newly-synthesised DA (see review by Glowinski, 1975, for details and references). More recently, Groppetti *et al.* (1977) by detailed analyses of the specific activity of DA and its metabolites soon after (5 min) *in vivo* (intraventricular) administration of ^{3}H-tyrosine, found that the specific activity of the metabolites of DA was higher than that of DA, indicating that they were derived from separate pools. Shore and Dorris (1975) found that short exposure to AMPT (30 min) markedly potentiated the haloperidol induced catalepsy, even though 80% of striatal DA was still intact; again suggesting that newly-synthesised (releasable) DA is preferentially released and that release from the large stable (stored) compartment is inadequate in the presence of haloperidol.

Although not complete, available data suggests that there are at least two "pools" of DA within the nerve terminal; a "releasable pool" into which newly-synthesised and recently taken up DA initially enter, and from which there is preferential release; and a "storage pool" which only slowly equilibrates with the releasable pool (Shore, 1976; see Figure 1.3).

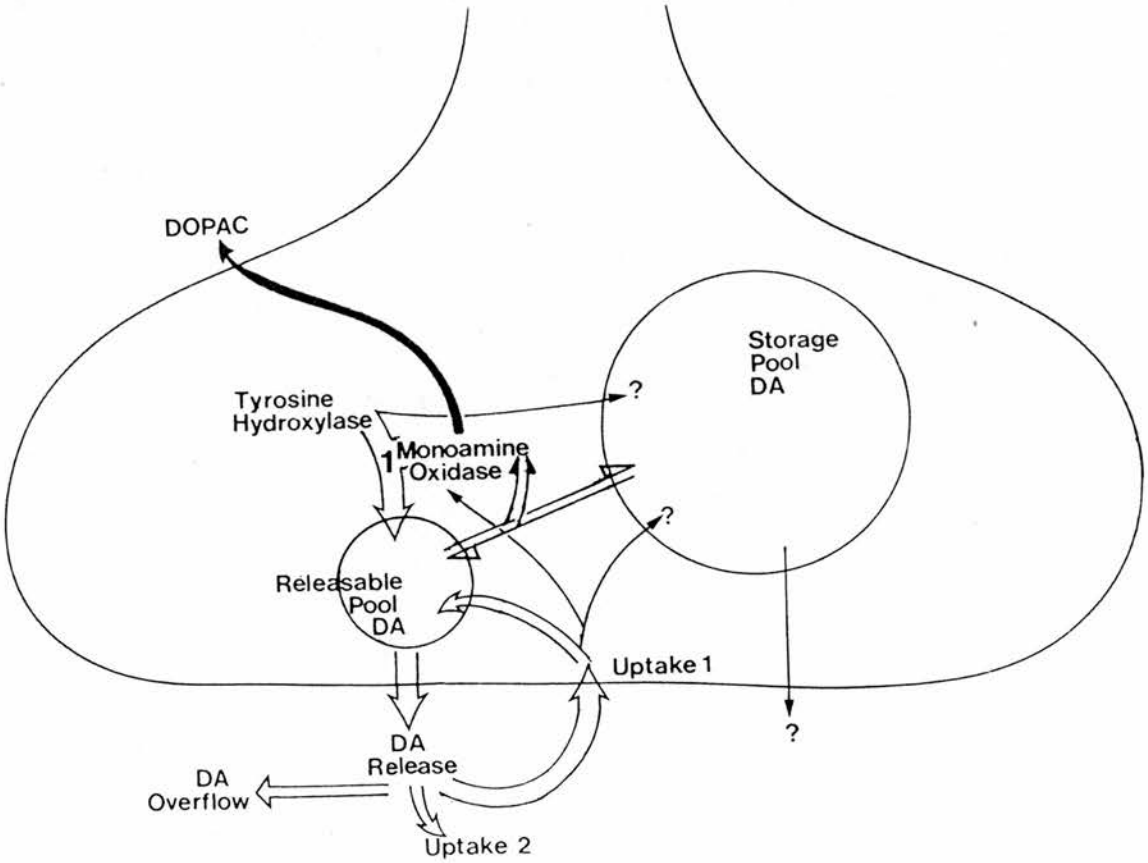
The assumption of a small releasable pool of DA, implies that changes in the synthesis rate of DA induced by drugs, would have a marked effect on the specific activity of DA in the releasable pool and hence on the specific activity of the overflow of total DA. Therefore, the release of ^{3}H-DA previously taken up by the tissue can at best

FIGURE 1.3: Schematic representation of the intraneuronal flux of dopamine.

The relative size of the releasable and storage pools of DA (less than 1 : 4) remains to be established. Although the movement of DA between the two pools of DA appears to be slow, its precise rate has not been established.

Site 1 may be taken to represent a 'cytoplasmic' pool of DA.

DA - dopamine; DOPAC - dihydroxyphenylacetic acid; Uptake 1 - neuronal uptake; Uptake 2 - extraneuronal uptake.



represent only part of the actual release of endogenous DA, and in most cases may not accurately reflect drug induced changes in the release of endogenous DA.

Most of the evidence presented above was obtained from the striatum, and as pointed out earlier is unlikely to hold, in every respect, for other dopaminergic areas of the CNS. Although there is some evidence to suggest that NA in noradrenergic nerve terminals, also exists in separate pools (Glowinski, 1975), evidence gathered by McMillen *et al.*, (1980) suggests that the separate pools of NA, unlike those of DA, are in rapid equilibrium, thereby more closely approximate a one-pool system.

1.3.2:

Central noradrenergic nerve terminals, like peripheral ones, appear to be endowed with presynaptic α-adrenoreceptors, stimulation of which results in the feedback inhibition of evoked NA release. This was first demonstrated by Farnebo and Hamberger (1971b) and has since been confirmed for electrically induced and K^+ induced overflow from both slice and synaptosomal preparations (Starke, 1973; Pelayo *et al.*, 1978; Mulder *et al.*, 1979; Baumann and Koella, 1980; Wemer *et al.*, 1981). Taube *et al.*,(1977) showed that the decreases and increases in ^3H overflow caused by α-agonists and α-antagonists respectively, was reflected in decreases and increases in ^3H-NA and its major metabolite ^3H-DOPEG overflow. α-Adrenergic agonists and antagonists have also been shown to decrease and increase, respectively, the 'turnover' of NA in the CNS (Anden and Garbowska, 1977; Anden, 1980).

The *turnover* of a neurotransmitter is generally defined as the rate of synthesis and/or utilization of the transmitter. Usually these are assumed to be equal, resulting in a more or less constant 'pool size' of the transmitter within the tissues. With increasing evidence for more than one pool of neurotransmitter within the presynaptic nerve terminal, and preferential release from a small (>20%) newly-synthesized pool (see above, Uvnas and Aborg, 1980) some reservations about the validity of turnover measurements has been expressed (Sharman, 1981). It appears therefore that the measurement of turnover after dopamine synthesis inhibition is more likely to be indicative of the rate of exchange between the storage and releasable pools; other methods used in turnover measurement (Westerink, 1979b; Sharman, 1981) are likely to be complicated by the disruption of the dynamic equilibrium of synthesis and utilization, and post-mortem changes (Carlsson *et al.*, 1974; Moleman *et al.*, 1977). These 'turnover' measurements are nevertheless useful indicators of a change in the rate of synthesis/utilization of neurotransmitters.

Neuroleptics have been shown to increase the turnover of NA in the CNS (Anden *et al.*, 1970; Nyback and Sedvall, 1970), and despite potentiation of basal release, neuroleptics also increase the evoked release of ^{3}H-NA from cerebral cortex slices (Farnebo and Hamberger, 1971b; Gorь and Schumann, 1980). Although Arbilla *et al.*,(1978) similarly found increases in the basal release of ^{3}H-NA with neuroleptics, the effect of neuroleptics on K^{+} induced release, in the presence of pargyline, was generally found to be facilitatory at low doses but inhibitory at high doses.

Dopaminergic presynaptic receptors

Dopaminergic presynaptic receptors have been reported to be facilitatory on 5-HT release (Reubi *et al.*, 1978; Cox *et al.*, 1980) but inhibitory on glutamate release (Mitchell and Doggett, 1980; Rowlands and Roberts, 1980), while both facilitatory (Reubi *et al.*, 1977), and inhibitory (Brase, 1980; Van Der Heyden *et al.*, 1980), dopaminergic presynaptic receptors have been described regulating the release of GABA.

There appears to be general agreement in the literature that the release of ACh from the striatum can be inhibited by dopaminergic presynaptic receptors (Miller and Friedhoff, 1979; Bianchi *et al.*, 1979; Stoof *et al.*, 1979; De Belleroche *et al.*, 1982; Scatton, 1982). An inhibitory influence of dopaminergic presynaptic receptors on ACh turnover has also been demonstrated (Guyenet *et al.*, 1975; Trabucchi *et al.*, 1975; Scatton, 1982). Hertting *et al.*,(1980) showed that ACh release from the striatum could be depressed by endogenous DA and suggested that the dopaminergic receptor subtype involved (Seeman, 1982) is of the D_2 type (see also Sethy, 1979; Scatton, 1982, Helmreich, 1981; Starke and Adelung, 1982). Destruction of the dopaminergic nerve terminals or chronic inhibition of the dopaminergic receptors appears to lead to a supersensitivity of the dopaminergic presynaptic receptors on cholinergic neurons (Stoof *et al.*, 1979; Miller and Friedhoff, 1979a).

Presynaptic modulation of dopamine release and turnover

In view of the methodological considerations set out above (1.3.1), it seems hardly surprising that a great deal of controversy surrounds almost every aspect of the control of DA release by presynaptic receptors (see Table 1).

TABLE 1: Summary of presynaptic receptors on dopaminergic neurons in the central nervous system.

Presynaptic receptors	Effect		References
Benzodiazepine	Release	+	Mitchell and Martin, 1980
	Turnover	-	Taylor and Laverty, 1973 Biswas and Carlsson, 1979
Prolactin	Release	+	Perkins and Westfall, 1978 [a] Gudelsky and Porter, 1980 [a] Foreman and Porter, 1981
	Turnover	+	[a] Johnston *et al.*, 1980 [a] Gudelsky and Porter, 1980
Prostaglandin E_2	Release	+	Baldessarini, 1975 Bergstrom *et al.*, 1973
	Turnover	-	Bergstrom *et al.*, 1973
Angiotensin II	Release	+	√Simonnett and Giorguieff-Chesselet, 1979
Adenosine	Release	-	Harms *et al.*, 1979 Michaelis *et al.*, 1979
Glycine	Release	-	√Giorguieff-Chesselet *et al.*, 1979 *Kerwin and Pycock, 1979 √*Roberts and Anderson, 1979 Martin and Mitchell, 1980
Glutamate	Release	+	√*Roberts and Anderson, 1979 √Giorguieff *et al.*, 1977b
	Release	0	√*De Belleroche and Bradford, 1980
GABA	Release	+	√Giorguieff-Chesselet, 1979b *Kervin and Pycock, 1979 √*Roberts and Anderson, 1979 *Starr, 1979 Stoof *et al.*, 1979 a*Sharman *et al.*, 1982
	Release	-	Bartholini, 1980 *Bowery *et al.*, 1980 Reimann, 1981

Table 1 (cont.)

Presynaptic receptors	Effect		References
Opiates	Release	-	*Loh *et al.*, 1976 Subramanian *et al.*, 1977
	Release	0	Arbilla and Langer, 1978 Bosse and Kuschinsky, 1978 Starr, 1978
	Release	+	√Gauchy *et al.*, 1973 Chesselet *et al.*, 1981
	Turnover	+	Laverty and Sharman, 1965 Gauchy *et al.*, 1973 [a] Deyo *et al.*, 1979 Moleman and Bruinvels, 1979 Wood *et al.*, 1980
ACh, Nicotinic	Release	+	√Giorguieff *et al.*, 1976, 1977b, 1979a √De Belleroche and Bradford, 1978
ACh, Muscarinic	Release	+	Lloyd and Bartholini, 1975 √Giorguieff *et al.*, 1977b [a] Perkins and Westfall, 1979 Bartholini, 1980 [b] *De Belleroche and Gardiner, 1982 Marchi *et al.*, 1982
	Release	-	Westfall, 1974a,b,c √*De Belleroche and Bradford, 1978
	Turnover	+	Laverty and Sharman, 1965 Anden and Bedard, 1971 Perez-Curet *et al.*, 1971 Nose and Takemoto, 1974 Javoy *et al.*, 1975 [c] Paalzov and Paalzov, 1975 De Belleroche and Bradford, 1980
5-HT	Release	-	[a] Cox *et al.*, 1980 *Ennis *et al.*, 1981
	Turnover	-	Andrews *et al.*, 1978 De Belleroche and Bradford, 1980

Table 1 (cont.)

Presynaptic receptors	Effect		References
Dopamine	Release	+	*Seeman and Lee, 1975, 1977 *Dismukes and Mulder, 1977 *De Belleroche and Bradford, 1981
	Release	0	*Raiteri *et al.*, 1978a,b, 1979 *Arbilla *et al.*, 1978
	Release	-	Farnebo and Hamberger, 1971 Lloyd and Bartholini, 1975 Plotsky *et al.*, 1977 Starke *et al.*, 1978 Reimann *et al.*, 1979 Jackish *et al.*, 1980 Arbilla *et al.*, 1981 [d]Dubocovich and Weiner, 1981
	Turnover	-	Carlsson and Lindqvist, 1963 Laverty and Sharman, 1965 Sharman, 1966, 1967 O'Keeffe *et al.*, 1970 Kehr *et al.*, 1972 Christiansen and Squires, 1974 Walters and Roth, 1974, 1976 Iversen *et al.*, 1975 Westfall *et al.*, 1976, 1979 DiChiara *et al.*, 1977 Garcia-Munoz *et al.*, 1977 Nowycky and Roth, 1978 Gale, 1979 Kapatos and Zigmond, 1979 Kehr and Debus, 1979 Bannon *et al.*, 1980 Haubrich and Pifueger, 1982

Notations:

√ - experiments during which only basal overflow was measured

* - experiments conducted in the presence of a monoamine oxidase inhibitor

\+ - facilitatory effects

\- - inhibitory effects

0 - no change

Striatal tissue was used in all reports with the following exceptions:

a - medial basal hypothalamus
b - nucleus accumbens
c - cortex
d - retina

While the effects of drugs on DA turnover are generally agreed upon, effects on release remain ambiguous. For example, while the facilitatory effects of opiates on DA turnover have been known for some time (see Table 1), such drugs have been demonstrated to have either no effect, or inhibit the release of ^{3}H-DA from striatal slices. Although Anden and Garbowska-Anden (1978) suggested that this may be because opiate induced increases in DA turnover are mediated from outside the striatum, *in vivo* experiments by Chesselet *et al*.,(1981) demonstrated that local infusion of morphine into the striatum increases the overflow of newly-synthesized DA. An *in vitro* increase in DA synthesis (from double labelled tyrosine) and basal release was also demonstrated by Gauchy *et al*.,(1973) supporting the view that opiates have a direct effect on dopaminergic nerve terminals of the striatum. The initial observation of Anden and Garbowska (1976) on a yohimbine (α_2-adrenoceptor antagonist) mediated increase in DA turnover, has recently been shown to be mediated by an action of the drug on dopaminergic rather than α_2-adrenergic presynaptic receptors (Scatton *et al*., 1980; Dedek *et al*., 1982), it is suggested therefore that striatal dopaminergic nerve terminals do not have (functional) α_2-adrenergic presynaptic receptors.

Despite ample evidence suggesting an increase in DA turnover and utilization by muscarinic agents *in vivo*, the effects of these agents on the *in vitro* release of ^{3}H-DA remains confused (see Table 1). Much of the confusion arises because the experiments show nicotinic or muscarinic effects on basal release only, the physiological significance and mechanism of which remains obscure. This confusion is compounded by the fact that during these experiments (Giorguieff *et al*., 1976, 1977a, 1979a; de Belleroche and Bradford, 1978) the

effect of drugs was examined on the basal release of newly-synthesized DA (which may be preferentially released), hence the possibility of an increased specific activity of a constant basal release (secondary to an increase in turnover) was not excluded.

Striatal dopaminergic neurons

i) Feedback regulation of dopamine turnover:

The feedback regulation of DA turnover has been known since the 1960's (Carlsson and Lindqvist, 1963; Sharman, 1966). The early suggestion of Carlsson and Lindqvist (1963) that the changes in DA turnover were mediated by a neuronal feedback loop (the striatonigral pathway, see Wright *et al.*, 1977) still has some support (Gale *et al.*, 1978; Gale, 1979). However, evidence gathered by Walters and Roth (1976a) and Kehr *et al.*, (1972) after inhibition of impulse flow in the nigrostriatal pathway, after lesioning the striatonigral pathway, Garcia-Munoz *et al.*, (1977) and DiChiara *et al.*, (1977) and *in vitro* work by Westfall *et al.*, (1976a, 1979) showed that despite effective isolation of the nigrostriatal terminals from effects mediated via the output pathway the actions of dopaminergic agonists and antagonists on DA turnover persist; suggesting that the effects are mediated, at least in part, by presynaptic dopaminergic receptors (see reviews by Nowycky and Roth, 1978; Westerink, 1979b). It is important to note that when impulse flow is inhibited in the nigrostriatal pathway (by lesioning or chemically) there is a paradoxical increase in DA turnover (Carlsson *et al.*, 1972; Kehr *et al.*, 1972). Hence neuroleptics are effective in inhibiting the actions of dopaminergic agonists on DA turnover (i.e. disinhibition of the decreased DA turnover), both *in vivo* after impulse inhibition (Kehr *et al.*, 1972; Walters and Roth, 1974, 1976a; Kehr and Debus, 1979), and *in vitro* (Christiansen and

Squires, 1974; Iversen *et al.*, 1976; Westfall *et al.*, 1979; Haubrich and Plfueger, 1982) while remaining largely ineffective when administered alone under these conditions. This suggests that when impulse flow is inhibited there is no release of DA and so presynaptic receptors are not activated, hence DA turnover is markedly increased (Nowycky and Roth, 1978). Support for this suggestion comes from the findings that neuroleptics are effective in increasing DA turnover *in vitro*, if tested during high K^+ depolarization (Westfall *et al.*, 1976a), or if the neuroleptics are administered *in vivo* initially (Goldstein *et al.*, 1973; Kapatos and Zigmond, 1979).

While there is growing evidence for a presynaptic dopamine receptor supersensitivity following chronic administration of neuroleptics (Nowycky and Roth, 1977; Skirboll *et al.*, 1979; Bannon *et al.*, 1980) and subsensitivity after chronic antidepressants (Serra *et al.*, 1979; Chiodo and Antl man, 1980) or dopaminergic agonists (Muller and Seeman, 1979); the suggested involvement of cAMP as a second messenger mediating the effects of the presynaptic receptor (Roth *et al.*, 1975) remain in doubt (Kehr and Debus, 1979; Anagnoste *et al.*, 1974). Although all these workers showed that cAMP does elevate the turnover of DA (see also Ebstein *et al.*, 1974), dopaminergic agonists which decrease DA turnover are associated with an increase in cAMP formation via the D_1 receptor (Kebabian and Calne, 1979; Seeman, 1981). Recent findings, however, suggest that the presynaptic effects of drugs acting at dopaminergic receptors are mediated by a D_2 type receptor (Helmreich, 1981; Starke and Adelung, 1982; Scatton, 1982), and most interestingly these D_2 sites have been shown to decrease cAMP formation after stimulation of adenylate cyclase with D_1 agonists in the striatum (Stoof and Kebabian, 1981) or isoprenaline (Tsuruta, 1981), *in vitro*.

ii) Feedback regulation of dopamine release:

After the initial observation of Farnebo and Hamberger (1971b) that the neuroleptic, chlorpromazine facilitates the evoked release of ^{3}H-DA, several authors have reported either no effect or an inhibition of release of ^{3}H-DA (Seeman and Lee, 1975; Dismukes and Mulder, 1977; de Belleroche and Bradford, 1981) with classical neuroleptics.

The evidence for facilitatory release modulating DA presynaptic receptors is based on the effects of neuroleptics on ^{3}H-DA release, it was usually found that DA agonists were ineffective in modulating the overflow of ^{3}H-DA (Dismukes and Mulder, 1977; Raiteri *et al.*, 1977a,b, 1979). Similarly (in the presence of MAOI), Miller and Friedhoff (1979b) found no consistent effect of apomorphine on ^{3}H-DA overflow, however, they did report a biphasic effect of haloperidol on ^{3}H-DA overflow, being stimulatory at low doses but inhibitory at high doses. While Seeman and Lee (1975) found fluphenazine to be more active than haloperidol in inhibiting the release of ^{3}H-DA, Dismukes and Mulder (1977) reported it to be inactive, however, Westfall *et al.*,(1976a) found that fluphenazine increased the synthesis and release of ^{3}H-DA, newly synthesized from ^{3}H-tyrosine, at a concentration of 10^{-6}M.

There is now increasing evidence for inhibitory release modulating dopaminergic presynaptic receptors, mainly from the laboratories of Langer and Starke (see Table 1), inhibiting the release of ^{3}H-DA (notably, neuroleptics are used in low doses and in the absence of MAOI). The most likely explanation for the discrepancies in the literature, therefore, is that as suggested earlier, ^{3}H-DA overflow is a poor index of endogenous DA release; the use of MAOI and high

concentrations of ascorbate (Lehmann *et al.*, 1981) makes a bad situation worse! Note that during experiments in which endogenous DA release (*in vivo*, Lloyd and Bartholini, 1975; or *in vitro*, Plotsky *et al.*, 1977) or the release of newly-synthesized-DA (Westfall *et al.*, 1976a) is measured, neuroleptics have been found to be facilitatory on DA release.

1.3.3 Background and aims of present investigation

The advent of the very sensitive catechol-O-methyl transferase radioenzymatic (COMT-RE) (Coyle and Henry, 1973) allowed the assay of endogenous catecholamines released from brain slice and synaptosomal preparations (Arnold *et al.*, 1977; Lane and Arison, 1977; Meyerhoff and Kant, 1978), however because COMT-RE is tedious, time-consuming and expensive, it was generally not used for this purpose. Similar considerations concerning other highly sensitive techniques (e.g. gas chromatography coupled with mass spectrometry [GCMS]) restricted most workers to studying the release of radiolabelled transmitters, which had been used so successfully to study neurotransmitter uptake (Iversen, 1975).

Recent advances in the development of high performance (pressure) liquid chromatography (HPLC) (see Knox, 1979), combined with electrochemical detection (HPLC-ECD), has provided a technique that is more suitable to the study of the release and metabolism of endogenous transmitters. "Soap-chromatography" or reverse-phase ion-pair exchange chromatography recently developed by Knox and Jurand (1976), is a technique which uses a reversed phase packing material in combination with a hydrophilic eluent containing an organic modifier (e.g. 10-15% methanol) and a small concentration of a detergent (e.g. 0.1%

sodium octyl sulphonate) which acts as an ion-pair reagent. The detergent appears to act in two ways to improve the efficiency of the column: 1) the detergent is adsorbed by the reversed-phase surface to form a layer which is in some ways similar to an ion-exchanger; 2) the detergent chosen has the potential to form an ion-pair with an ionized form of the solute (the pH is controlled to ensure that the solutes of interest are ionized). Thus for amines an acidic eluent containing a cationic detergent is generally used. The ion-pairs formed, being more hydrophobic, dissolve in the stationary phase to achieve separation. Hence both catecholamines and their metabolites can be separated in one step (Felice *et al.*, 1978).

The electrochemical detector, refined and applied to catecholamine assay by Kissinger and Adams (Kissinger *et al.*, 1973), works on the following principle: From Figure 1.4, the variable power source, connected through an operational amplifier (OP amp) to the reference (Y) and auxiliary (R) electrode is used to maintain a predetermined constant voltage at the reference electrode. A potential difference is then established between the surface of the working electrode (B) (maintained at virtual zero) and the solvent (i.e. the mobile phase of the HPLC) in the low volume chamber. Any electroactive species, oxidisable at or below the potential set up at the reference electrode, is oxidized, as shown in Figure 1.4 for dopamine, which then liberates protons and electrons. The electrons flow down the potential gradient into the working electrode (involving a phase transfer for the electron), the current is converted to a voltage capable of driving a chart recorder. The general expression developed by Levich (1962) for this current at an electrode under forced convection conditions (e.g. in a flowing stream; amperometric detectors in stirred solutions;

FIGURE 1.4: A. Skeleton circuit of the electrochemical detector.

-15V and +15V = operational amplifier regulated power source.

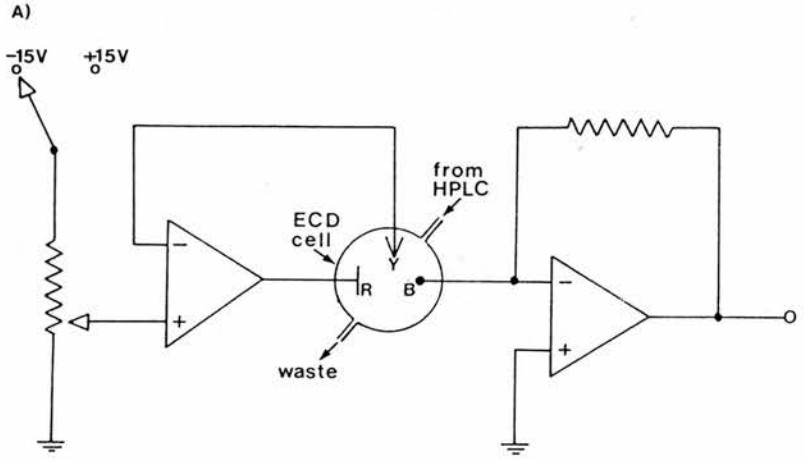
A_1 and A_2 = operation amplifiers.

ECD cell = electrochemical detector cell.

Y = reference; R = auxiliary; B = working electrodes.

B. Levich (1962) equation for the limiting current at an electrode under forced convection conditions. Where i_L = limiting current obtainable from oxidation or reduction of a compound; K = cell constant which depends on the diffusion coefficient of the compound of interest, the kinmetic viscosity of the solvent and the geometry and area of the electrode; n = number of electrons involved in oxidation/reduction of one molecule of the compound of interest; F = Faraday's constant; V^a = velocity of the solution passing over the electrode to some power, a; C_b = bulk concentration of the compound present in solution. All else being constant i_L becomes directly proportional to C_b.

C. The oxidative mechanism for catecholamines. Dopamine is oxidized to a quinone liberating two electrons and two protons.



rotating disc electrodes) is also shown in Figure 1.4. Note that under isocratic chromatographic conditions at a constant flow rate and temperature (since the diffusion constants and viscosity, represented by K, are temperature dependent), the current obtained, i_L, will depend only on the concentration of the electroactive species in solution C_b.

Using a modification of the electrochemical detector designed by Kissinger *et al.*, (1973) and Keller *et al.*, (1976), and reverse-phase ion-pair chromatography, a protocol for studying the release and metabolism of endogenous DA, *in vitro*, was established. A series of experiments was carried out to examine the influence of cholinergic and dopaminergic mechanisms regulating the synthesis and release of DA.

CHAPTER II

Materials and Methods

2.1 Electrochemical detection

Amperometric (electrochemical) detector

Figure 2.1 shows the circuit diagram of the amperometric detector, modified after Keller *et al.* (1976) and Kissinger *et al.* (1973). The points marked +15V and -15V are connected to a standard operational amplifier (op-amp) regulated power supply (Farnell). The circuit contains three op-amps, A1, 2 and 3 (Analog Devices), which form the basis of the three part circuit:

A1 - constant voltage source, maintaining a constant voltage at the reference electrode.

A2 - current to voltage converter, for the current picked up by the working electrode.

A3 - amplifies the voltage from A2, and controls the gain and offset - null.

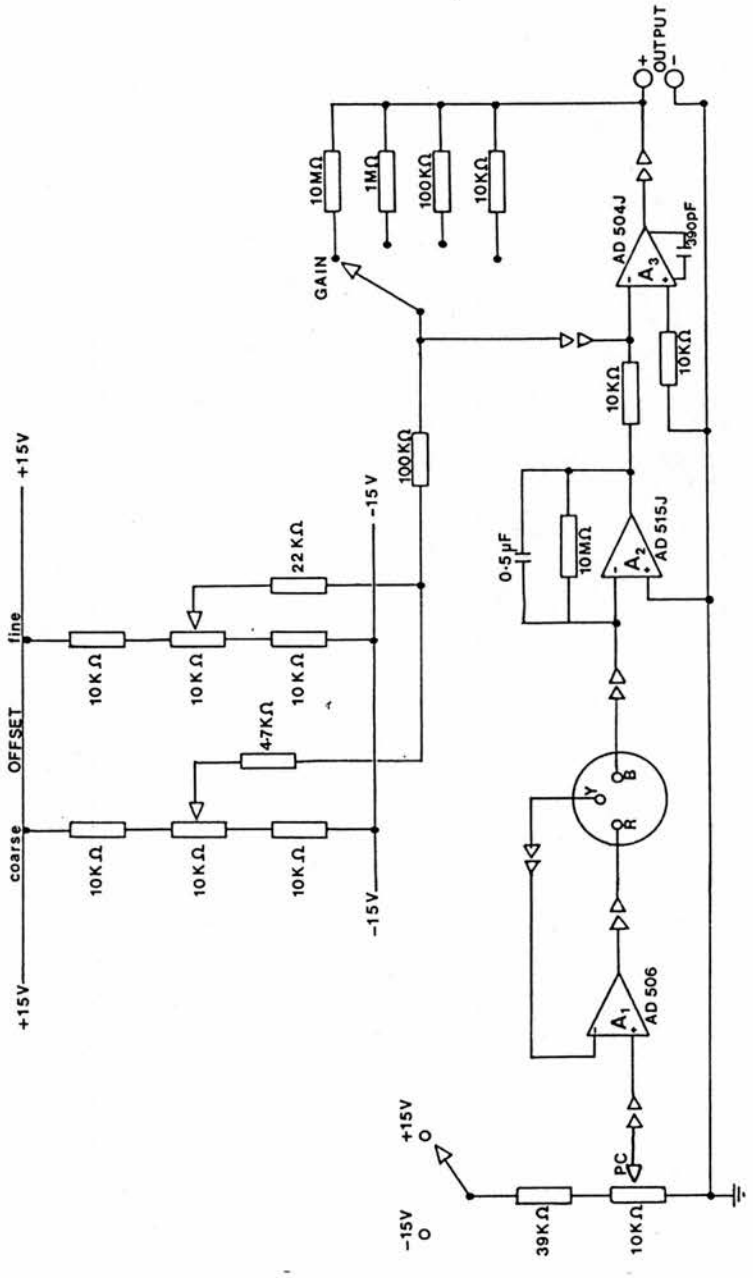
The detector was housed in a small aluminium box and the output fed to a two-channel Bryans strip chart recorder (Model BS600). Great care was taken to ensure that everything including the HPLC was properly earthed and screened leads were used to connect the detector cell to the amperometric detector, to reduce background noise as much as possible.

Briefly the setting up procedure was as follows. With the polarity switch at -15V, the reference and auxiliary electrodes were connected to the detector. The potential control potentiometer was adjusted so as to give the desired voltage (0.5 - 0.75V) between the reference electrode and earth (0 V). With the gain setting at 100nA/V, the working electrode was allowed 60-90 min to settle down before any injections were made. Using the offset potentiometers, the background current was nulled out and several test injections were made to test the electrode sensitivity and stability. The amperometric detector was normally left

FIGURE 2.1: Circuit diagram of the electrochemical detector.

The points marked +15V and -15V are connected to a standard operation amplifier controlled power supply, with PC being the potential control potentiometer. Gain and offset null controls are as marked.

Connections to the electrochemical detector cell were made at Y = Reference; R = Auxiliary; B = working electrodes.



switched on overnight at a very low HPLC flow rate (0.1 ml/min) to minimize setting up and electrode "settling down" time. This did not appear to have any adverse effect on the sensitivity or operational life of the detector cell.

Detector cell

Perspex detector cell blocks of the same basic design as Kissinger *et al.*,(1973) were made, as shown in Figure 2.2. In some later experiments, the Bioanalytical systems plexiglas thin layer detector cell (TL3) was used, the only difference being that the working electrode was in the centre of the lower block of the cell, and a small metal outlet tube acted as the auxiliary electrode in the reference/auxiliary electrode compartment.

The most important point in the care of the detector cell was to avoid scratching in the inner surface of the perspex, and to ensure that the inlet/outlet holes, and the depression made for the working electrode, were carefully machined to give perfect edges. The surfaces of the blocks were polished by gently rubbing the blocks on wet paper towels and using a silver polish (Silvo, Reckitt and Colman Ltd.).

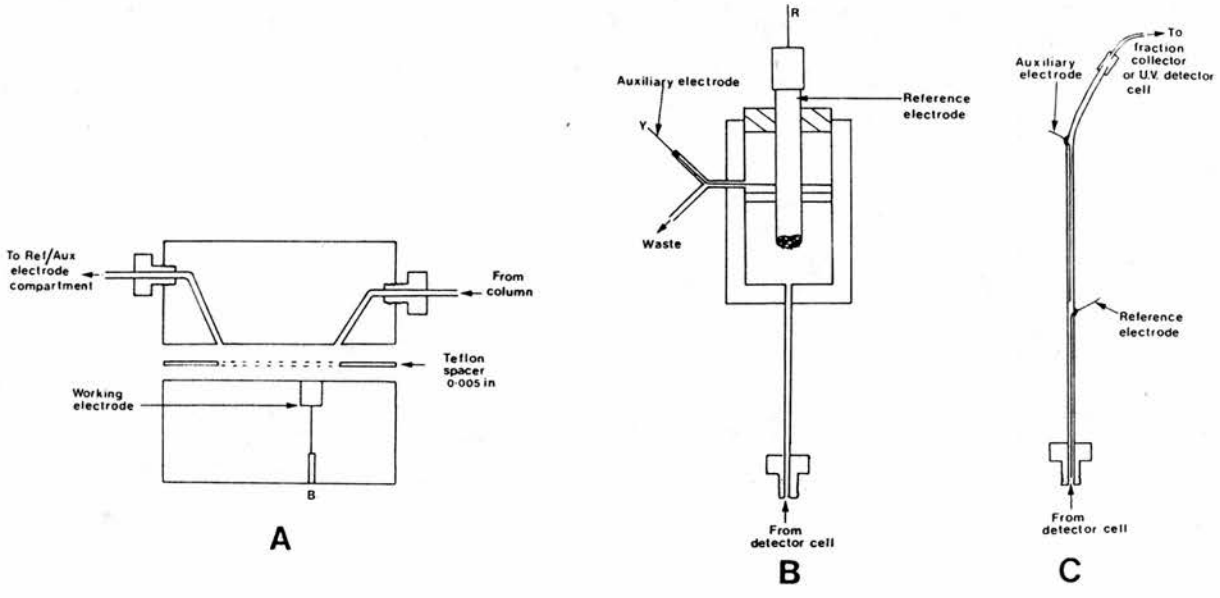
The carbon paste (CP-S) generally used was made up as follows: 3.25 gm of graphite powder (Koch-Light Labs Ltd.) + 1.75 g High Vacuum Grease (Edwards High Vacuum) were thoroughly mixed in a mortar and pestle to give uniform consistency. The graphite paste was tightly packed into the depression for the working electrode with a matchstick (when repacking, as much as possible of the old paste was removed). Sufficient paste was then gently pressed on to leave a small mound. The final electrode surface was formed by carefully rubbing the overpacked electrode block on 3-4 sheets of lens cleaning tissue placed on a clean glass sheet or tile. The electrode surface was

FIGURE 2.2: Detector cell and Reference/Auxiliary electrode design.

A. Detector cell made to the specification of Kissinger *et al.*, 1973.

B. Reference/Auxiliary electrode compartment. The calomel or Ag/AgCl reference electrode was held in a perspex box, using a rubber bung. A piece of silver wire, wound around the inside of the perspex box, acted as the auxiliary electrode.

C. Low dead volume Reference/Auxiliary electrode compartment. Chloridized silver wire pieces, inside a teflon tube ($\frac{1}{16}$") acted as the Reference and Auxiliary electrodes.



examined under a dissecting microscope and when free from scratches, and polished, any residual carbon paste around the working electrode was rubbed off the perspex block with some soft tissue paper soaked in ethanol. The CP-S paste was found to be generally easy to polish but took longer to equilibriate when new, however the response was the same as CP-O (Nujol-oil based graphite paste, Bioanalytical Systems) at +0.7V. CP-O allowed for slightly greater sensitivity at lower potentials (+0.55V) but was more difficult to polish in order to get an adequately "quiet" cell. CP-O was used in later experiments.

The reference electrodes used were calomel electrodes (Russell pH Ltd.) or the Ag/AgCl electrodes (RE1, Bioanalytical Systems). Chloridised silver wire (Ag/AgCl) electrodes were made by quickly dipping silver wire into molten silver chloride, while ensuring an even coating of the silver chloride onto the wire. Modifications to the reference/auxiliary compartment, depicted in Figure 2.2c, was especially designed to allow collection of the peaks immediately after detection. Dead space in the teflon tubing was less than 400µl.

During the setting up of a new cell, a common problem encountered (apart from an inadequately polished electrode surface) was air bubbles getting trapped in the detector cell. While these could generally be dislodged by connecting a syringe with a luer-adapter to the inlet port and flushing the system with buffer, to carry out this operation it was necessary to switch off the amperometric detector, with an inevitable delay when restarting to allow time for the electrode current to reach the basal level. Once set up, the detector cell and reference and auxiliary electrode compartments were covered with a wire cage which served as a "Faraday cage"; this was not always found to be necessary as the laboratory proved to be electrically quiet.

The response of the ECD cell to injected catecholamines was linear over a very large range (see Figure 2.3), and so could be easily calibrated to measure unknown amounts in injected samples.

2.2 High performance liquid chromatography (HPLC)

HPLC pumps

Initially, during the development of the amperometric detector, a Dupont 302, constant pressure HPLC pump was used. Since the pump was gas (N_2) operated it had the advantage of being electrically quiet; however, several major disadvantages outweighed this advantage. Firstly, this HPLC-pump was rather antiquated and proved to be difficult to set up and maintain in working order. Secondly, while the slowly wandering base line (due to changes in the flow rate) was not a limiting factor, whenever the pump primed itself, there was a very large transient drop in pressure and flow rate (piston volume 60 ml) which was not damped. This was particularly evident when working at the higher pressures required for ion-pair chromatography (1200 psi). Hence after the rather drastic fluctuations in the base line (due to the priming action of the pump every 2-3 injections) a considerable time had to be allowed for the base line to re-establish itself. Thirdly, because of inadequate degassing facilities, bubbles developing at the end of the HPLC column were forced into the detector cell causing great fluctuations in the base line and shortening the life of the working electrode surface, again the problem was worse when working at higher pressures. Fourthly, although the injection port with septum was adequate during tests with the ion-exchange column (working pressure = 450-500 psi), the stop-flow injection techniques had to be employed during ion-pair soap chromatography (working pressure = 1200-1500 psi) -

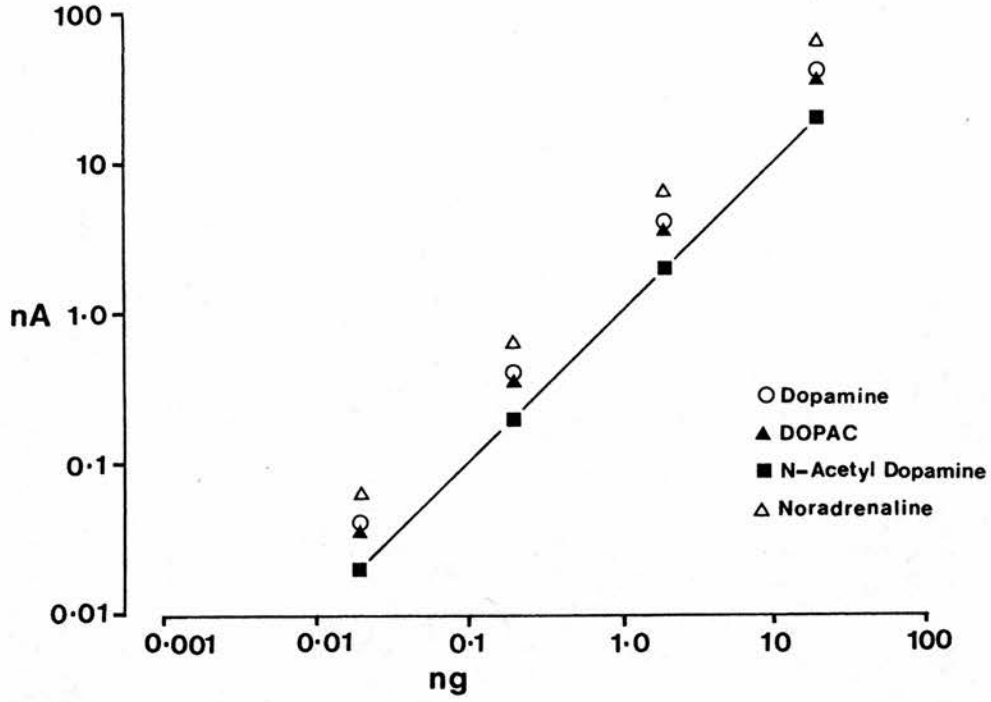


FIGURE 2.3: The range of linear response of the electrochemical detector for catecholamines, their metabolites and derivatives.

this of course caused fluctuations in the base line due to dramatic changes in pressure (see Figure 2.4).

The pump subsequently used was a Gilson 301 constant flow pump with a 10 ml pumping head which was far better suited for the purposes of electrochemical detection. Three different pulse damping mechanisms: 1) a small metal frit in the pump head just before the outlet check valve; 2) a long, empty column introduced into the flow; and 3) a built-in electronic compensator (regulating the time taken for the pump to prime) ensured an adequately pulse-free flow.

Difficulties with injecting samples at high working pressure were overcome by using injection valves (Rheodyne 7125 or Altex 210-20) normally using 20µl or 50µl sample loops. Figure 2.5 shows a flow diagram of the equipment used for the HPLC-ECD analysis of all superfusate samples.

HPLC column packing

A 25 cm Partisil SCX-10µ (Shandon) cation-ion exchange column was used for ion exchange chromatography.

Ion pair chromatography was carried out using stainless steel columns (25 cm) which were packed with C_{18} -reverse phase packing material (Shandon, Hypersil-ODS, 5µ particle size) using a Magnus P6050 slurry packing unit. The slurry, made up with 3-4 g packing material in 50 ml of 0.1M sodium acetate dissolved in HPLC grade methanol (Rathburn Chemicals, Walkerburn, Scotland) was poured into a small ultrasonic tank, to ensure that the packing material particles were kept separate and then forced into the column at a pressure of 5000 psi with degassed methanol. The columns were then washed thoroughly with methanol followed by 10% methanol in distilled and deionized water and subsequently allowed to equilibrate with the HPLC

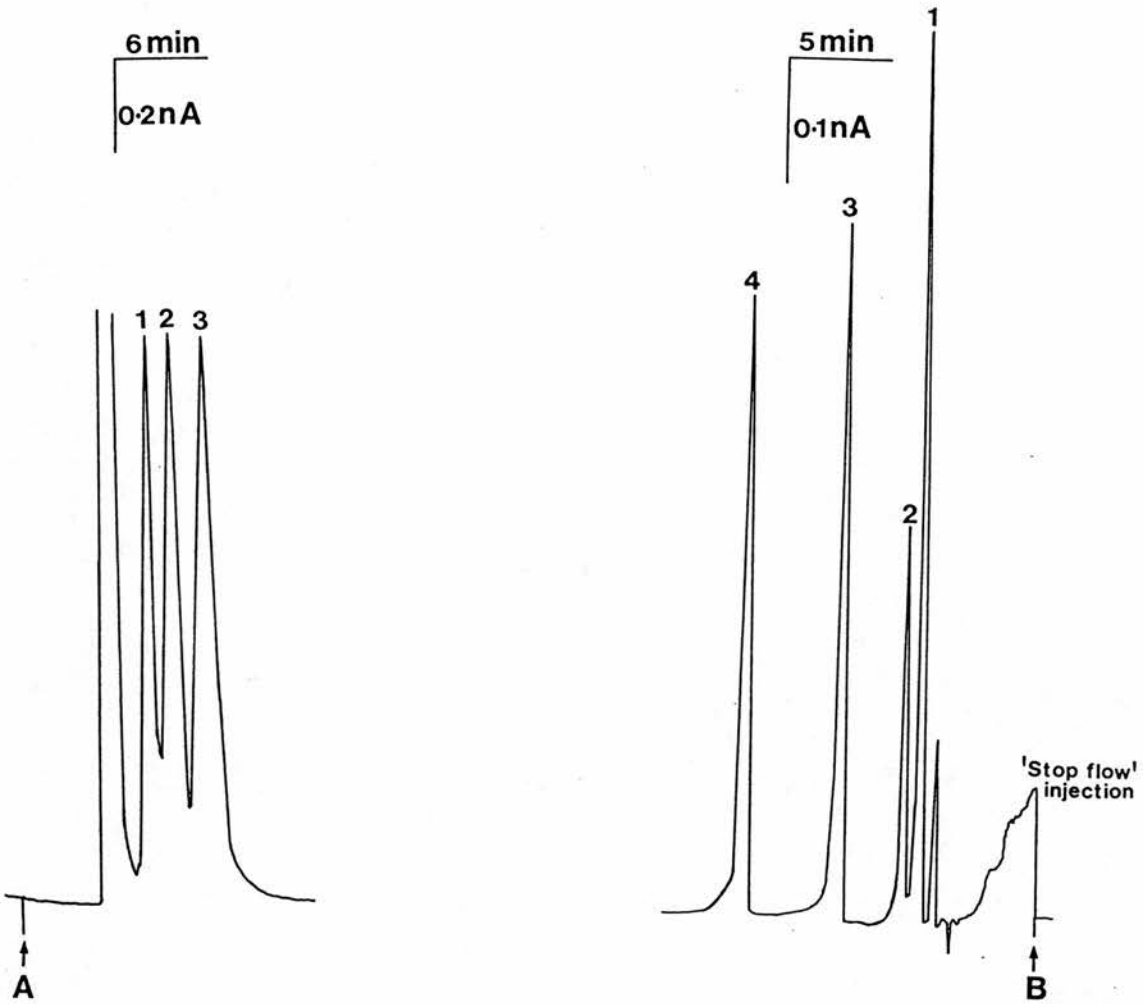


FIGURE 2.4: A. Ion-exchange separation of 1 = noradrenaline; 2 = adrenaline; 3 = dopamine.

B. Ion-pair separation of 1 = noradrenaline; 2 = adrenaline; 3 = dopamine; 4 = dihydroxy-phenylacetic acid.

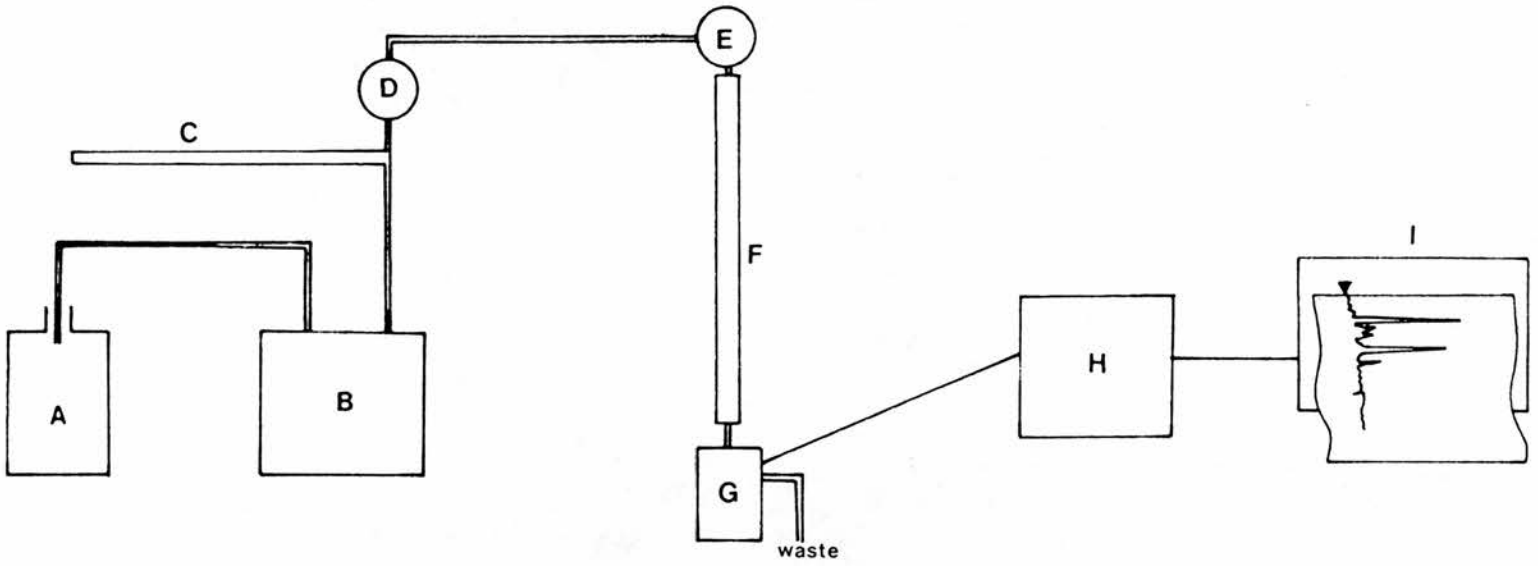


FIGURE 2.5: Flow diagram of the High Performance Liquid Chromatography with Electrochemical Detection analysis of catecholamines.

A = buffer; B = pump; C = pulse damper; D = pressure gauge; E = injection valve; F = C_{18}-reverse phase column; G = detector cell (see Figure 2.2); H = amperometric detector; I = chart recorder.

buffer (see below) for at least 12 h. The columns were tested (according to Knox, 1979) and only columns with more than 1000 theoretical number plates (N) were used, where

$$N = 16(t_R/W_t)^2$$

where N = number of theoretical plates
t_R = retention time
W_t = peak width (time units) at base.

Peak spreading or trailing, especially in dirty overused columns, could often be corrected by opening the top of the column, removing 1-2 mm of the packing material and replacing it with clean slurry. Because the test samples injected onto the columns did not contain high molecular weight, proteins, lipids etc, the columns were kept in continuous use for the life-span of the detector cell (3-4 weeks). The column was, at the time of changing the detector cell, washed (50 ml distilled water, followed by 50 ml methanol, 50 ml of a 1 : 1 mixture of methanol and chloroform, 50 ml of methanol and 50 ml of water, in that order) before re-use.

Buffer systems

The buffer system used for ion exchange chromatography was as described by Keller *et al.*, (1976) and made up as follows: (pH ca. 5.2)

Citric acid, $1H_2O$	- 5.75 g
Sodium acetate, $3H_2O$	- 6.80 g
Glacial acetic acid	- 1.05 ml
Sodium hydroxide	- 2.40 g

made up to 1 l with distilled and deionized water,
flow rate: 1 ml/min.

However, this form of separation was less efficient (see Figure 2.4) and because DOPAC cannot be measured directly, ion exchange chromatography was substituted by ion pair chromatography for all experimental purposes.

For reverse phase ion-pair chromatography the buffer used was made up as follows:

Potassium dihydrogen orthophosphate	-	13.61 g
Sodium octyl sulphonate	-	25.0 mg
EDTA	-	35.0 mg
HPLC grade methanol	-	140 ml

made up to 1 l with distilled and deionized water, and the pH adjusted to 4.0 with 1M citric acid.

Degassing of the buffers was done by first applying a vacuum for about 2 min, and then bubbling with Helium gas for 20-30 min.

The HPLC flow rate was set at 0.8 ml/min, giving retention times of approximately 7 min for DA and 10 min for DOPAC (see Figure 2.6). One great advantage of this type of chromatography was that small alterations of the buffer composition altered the retention times and the order of elution of the compounds. Retention times were increased by: a) increasing the amount of ion pair reagent; b) decreasing the methanol (organic modifier) content; c) altering the ionization state of the catecholamines by decreasing the pH. Since, for example, DOPAC with its acid group is more sensitive than catecholamines to pH changes, the position of DOPAC relative to DA could be altered by pH changes. Adding other ions to the buffer, e.g. sodium acetate had, likewise, the effect of decreasing the retention time of DOPAC greatly while leaving the DA retention time almost unaltered. While these relationships were not examined in detail, they were used to try to verify the identity of compounds, as two compounds are

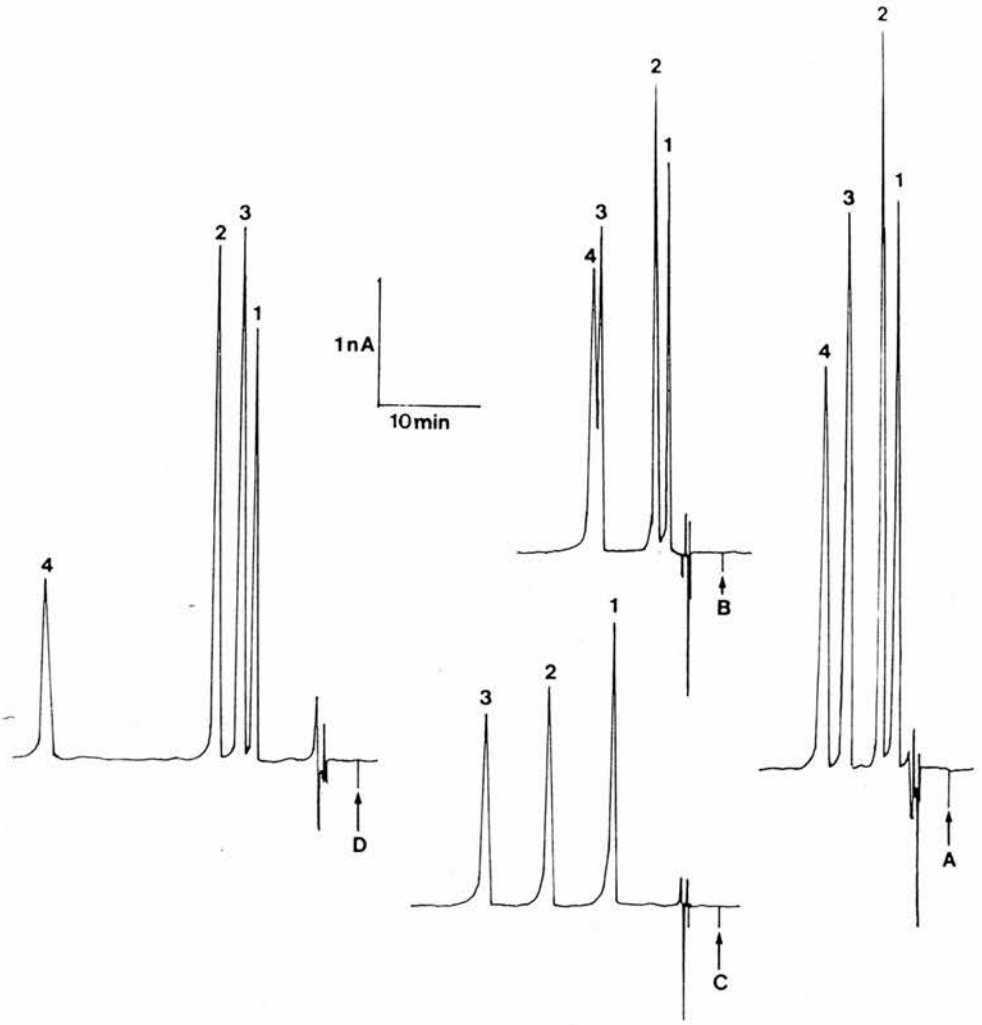


FIGURE 2.6: Ion pair separation of 1 = dihydroxybenzyl amine; 2 = dopamine; 3 = dihydroxyphenylacetic acid; 4 = 3-methoxytyramine, under different chromatographic conditions (flow rate 0.8 ml/min):

A. Standard buffer composition (see text). Amounts injected: 1, 2 and 3 = 2 ng; 4 = 4 ng.

B. Buffer composition as above except pH = 3.5. Amounts injected: 1, 2 and 3 = 1 ng; 4 = 2 ng.

C. Buffer composition as above except methanol reduced to 5%. Amounts injected: 1, 2 and 3 = 1 ng.

D. Buffer composition as above except sodium octyl sulphonate increased to 125 mg/l. Amounts injected: 1, 2 and 3 = 2 ng; 4 = 4 ng.

Note that decreasing the methanol content increased the retention time of all the compounds while changes in the pH only affected the retention time of the acid metabolite and changes in the ion-pair reagent only affected the amine retention times.

unlikely to have the same retention times under different chromatographic conditions (see Figure 2.6). Retention times of authentic compounds were checked before and at the end of every experiment, as was the sensitivity of the detector cell.

2.3 Comparison with other techniques

UV spectrophotometry

Using the low dead volume reference and auxiliary electrode compartments depicted in Figure 2.2c, the outflow from the detector cell was connected to a spectrophotometer (Perkin-Elmer, LC-55) and monitored at 280nM.

Gas chromatography-mass spectrometry (GCMS)

Samples for GCMS, containing 0.01mM ascorbate, were evaporated to dryness in a vacuum dessicator and then derivatized using heptaflurobutyric anhydride (HFB anhydride). The res ulting tri-HFB catecholamine derivatives were evaporated to dryness and redissolved in ethyl acetate and injected into the GCMS. The retention time of the tri-HFB-DA derivative on the GC was about 3 min. Using mass fragmentography with multiple ion detection, DA was identified and quantified (528, 331 and 226 m/e, fragments of the tri-HFB-DA were found, deuterated-DA was used as the internal standard).

2.4 Superfusion techniques

Superfusing solutions

For superfusion of striatal, median eminence and retinal tissue, Krebs buffer was used. Its composition was as follows:

	g/l
Sodium chloride	- 6.90
Sodium bicarbonate	- 2.10
Glucose	- 1.80
Potassium chloride	- 0.28
Magnesium sulphate	- 0.30
Potassium dihydrogen-orthophosphate	- 0.16
Ascorbic acid	- 0.02
Calcium chloride	- 1.75 ml of a 1M solution

pH ca. 7.4

High K^+ (15, 25, 35, 50mM K^+) Krebs was made up by substituting 10, 20, 30 or 45mM NaCl respectively, with the equivalent amount of potassium chloride.

Superfusion of cockroach salivary glands was carried out using a Ringer solution of the following composition.

Sodium chloride	- 9.35 g/l
Potassium chloride	- 1.00 ml of 1M solution
Calcium chloride	- 5.00 ml of 1M solution
Tris	- 0.605 gm/l
Hydrochloric acid	- 4.00 ml of 1M solution
Ascorbic acid	- 0.02 g/l

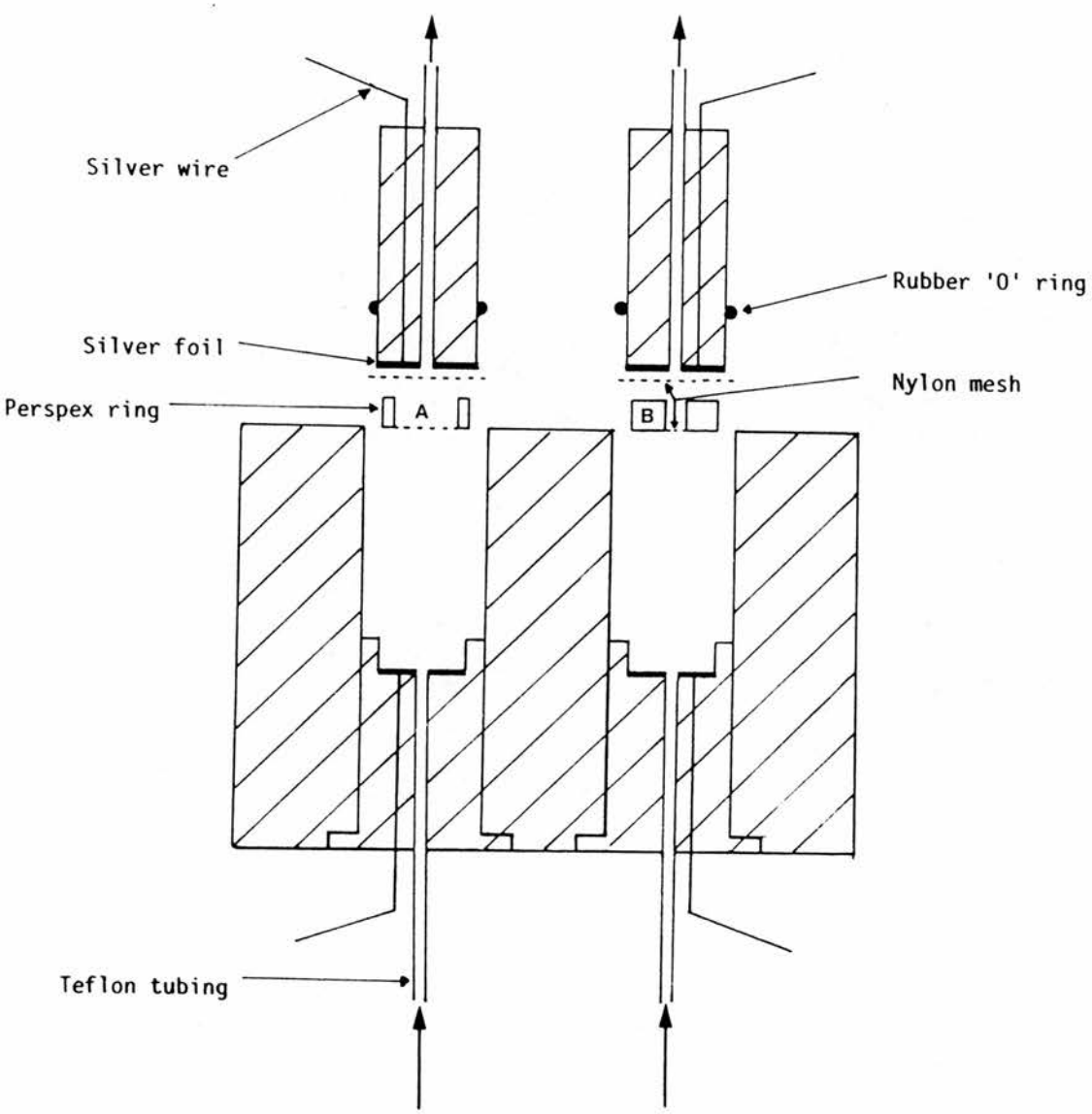
pH ca. 7.6

All superfusing solutions were continuously bubbled with 95% oxygen, 5% carbon dioxide throughout the course of the experiments.

Superfusion chambers

The superfusion chambers, shown in Figure 2.7, were made of perspex and used to run two experiments in parallel. Tissues were placed in the perspex ring (internal volume 200µl), within the superfusion chamber, covered with a nylon gauze and held in place by a perspex stopper which also served as the outlet port. At the end of

FIGURE 2.7: Schematic diagram of the superfusion chambers. Tissues were placed in the perspex rings (A for striatal and retinal tissue; B for median eminence and cockroach salivary glands) and covered with a nylon mesh. This was held in place by a perspex stopper which also served as the outlet part. Silver foil electrodes allowed for electrical stimulation of the tissue, the resistance between them during superfusion with Krebs or Ringers solution was 2-3KΩ. At the end of the experiments, the perspex rings were taken out and the tissue removed for protein estimation and/or tissue catecholamine content determination.



the experiment the perspex ring was taken out, the tissues removed, homogenized and assayed as necessary (see later).

For experiments with median eminence tissue pieces and cockroach salivary glands, a modified perspex ring was used; the internal volume was reduced to approximately 50µl because of the small amount of tissue, and to decrease the dead volume in the system.

Basic superfusion protocol

A flow diagram of the basic superfusion technique, common to all experiments, is shown in Figure 2.8. A Watson-Marlow peristaltic pump with a head attachment that allowed different solutions to be pumped simultaneously was used. Inlet tubes to the superfusion chambers were supplied with rubber tube collars (RC) which permitted water tight joints that could be quickly and easily interchanged, so that a tube containing high K^+ Krebs could be connected to the chambers for two minutes and then the original Krebs solution (+,- drugs) could be replaced to allow for a 2 min pulse of high K^+ Krebs. Unless otherwise stated, 25mM K^+ Krebs was used. This procedure was synchronized with the fraction collector (by measuring the flow rate and dead volume in the tubing), allowing the 2 min pulse of high K^+ to be collected as one sample. The fraction collector used was a Gilson microcol TDC 80, set to collect 2 min samples. Drug stock solutions were added directly to the Krebs (or Ringers) solutions.

As indicated in Figure 2.8, the superfusion chamber (and a length of inlet tubing) was immersed in a water bath at 37°C for all experiments except when working with cockroach salivary glands (these experiments were carried out at room temperature, 20°C). Two Digitimer isolated stimulators (Model DS2) were used to provide a biphasic square wave pulse for electrical stimulation. The stimulators

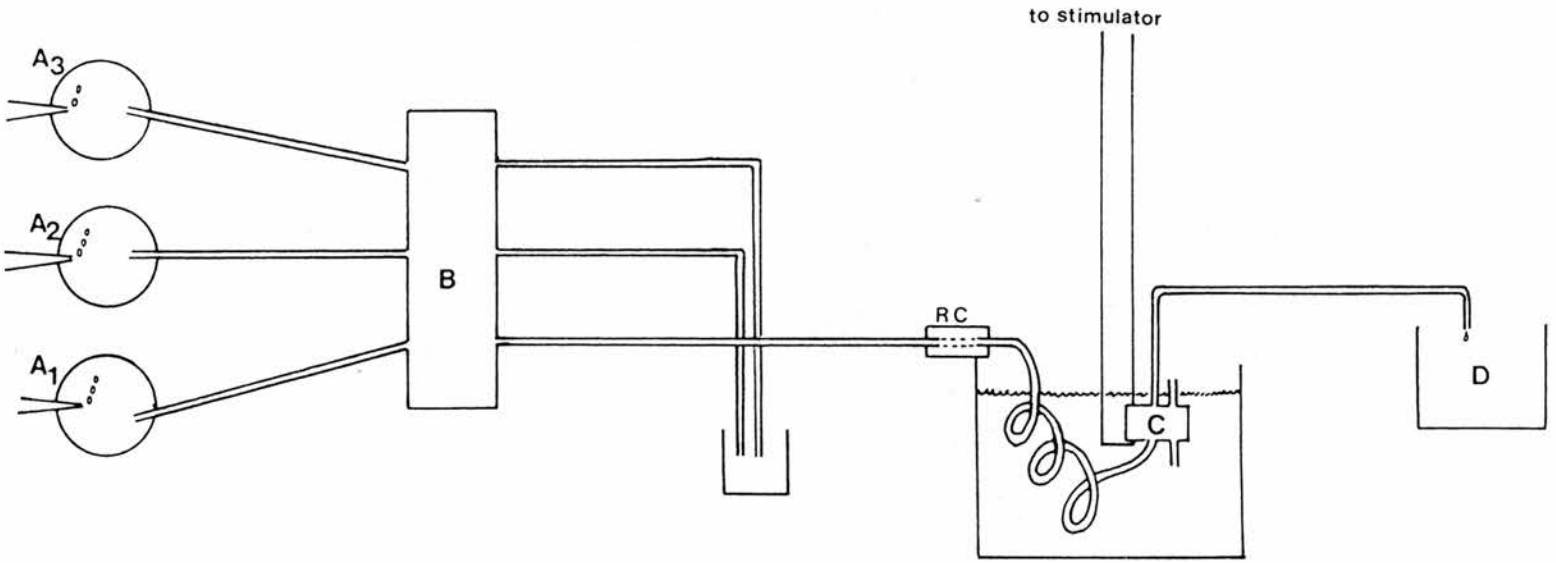


FIGURE 2.8: Flow diagram of the superfusion technique and the equipment employed.

$A_{1,2,3}$ = superfusing solutions and high K^+ ion concentration media, with or without drugs, being continuously bubbled with 95% oxygen, 5% carbondi oxide. B = peristaltic pump with a multi-tube head attachment. RC = rubber tube collar for rapid changing of tubes to allow for a 2 min pulse of high K^+ Krebs. C = superfusion chamber (see Figure 2.7) immersed in a water bath at 37°C and connected to the stimulator. D = fraction collector, set to collect 2 min samples into Eppendorf tubes at 0°C, containing 7µl of a protective solution + internal standard (see text).

were driven by a Digitimer D100 pulse generator and the output of the stimulators constantly monitored on an oscilloscope (Telequipment DM64) across a 500Ω resistor. Two silver rings in the superfusion chamber served as the stimulating electrodes. The resistance between these electrodes was between 2-3KΩ under experimental conditions. Unless otherwise stated, the stimulation parameters used were: 8-10mA amplitude, 2msec duration, at 20Hz for 30 sec.

Tissue dissection and details of superfusion

A) Striatal tissue: Male albino Wistar rats (180-220 gm) were used throughout. Rats were killed by cervical dislocation, decapitated, and the brains rapidly removed from the cranial cavity. The brains were placed in ice cold, oxygenated Krebs for about 20 sec and then placed, ventral side up, in a petri dish on ice. Two cuts were made, the first 1-2 mm anterior to the optic chiasma and the second just before the optic chiasma. From the resulting slice of brain tissue, both striata were dissected free and chopped (McIlwain tissue chopper) at 0.3 mm intervals, twice, at right angles. The tissue pieces were placed in 10 ml of oxygenated Krebs at 37°C, separated by one or two moderately violent shakes, and allowed to (equilibriate) preincubate for 10 min. The tissue was then transferred to the two superfusing chambers and superfused at approximately 300-350µl/min (the exact value being determined at the end of the experiment by averaging the volumes collected into the sample tubes).

During experiments with unilaterally lesioned animals, the lesioned and unlesioned striata were kept separate and put into different superfusing chambers.

After transferring the tissues to the superfusing chambers, they were allowed a 10 min wash-out period before collection of the Krebs

solution commenced. Normally, two periods of stimulation (using high K^+ Krebs or electrical stimulation as described) were applied during an experiment, the first (S_1) at 16 min, and the second (S_2) 56 min after the onset of superfusion. Drugs were applied 30 min after the onset of superfusion, i.e. after the control S_1. All the samples between 10-30 min and 50-70 min were analysed for their DA and DOPAC content.

At the end of the experiments the tissues were removed from the chambers (as described previously) and homogenized in 1 ml of 0.1M HCl (containing 0.01mM EDTA +.1µM Ascorbate) solution. A 30µl aliquot was immediately taken for protein estimation (Lowery *et al.*, 1951) and the rest spun down for 10 min at 12,000 rpm (Eppendorf 3200 centrifuge) at 4°C. 100µl of this supernatant was diluted 1 : 10 and injected directly into the HPLC-ECD for DA and DOPAC estimation. The DA/DOPAC content of the tissues at the start of the experiment was determined as above using a 3-4 mg (wet weight) sample of tissue taken just after the preincubation period.

During experiments with labelled DA, ^{3}H-DA was added to the 10 ml of Krebs solution during the preincubation stage. The slices were then washed three times with label-free Krebs before being transferred to the superfusion chambers. ^{3}H overflow was determined by pipetting 500µl of the superfusate into vials containing 8 ml of scintillant (Aquasol). The radioactivity in the samples was determined by Liquid Scintillation Counting (Searle, Mk.II, Nuclear Chicago Division). In one set of experiments (3.1) the eluent of the HPLC-ECD was collected, using the low-dead volume reference/auxilliary compartment. Radioactivity in these samples was determined as above. At the end of the superfusion experiments, 100µl of the tissue homogenate was used to determine the amount of radioactivity left in the tissue.

B) Median eminence tissue: The following alterations were made to the protocol mentioned above during experiments with median eminence tissue:

1) The chilled brain of the rat was placed in a petri-dish, on ice, under a dissecting microscope. With the aid of a green light source, the median eminence was identified using the hypothalamo-hypophysial portal blood vessels as a landmark. With the aid of a fine pair of forceps, the remaining part of the pituitary stalk was gently lifted and two incisions made, parallel to the blood vessels using the stalk as a guide. The incision was made up to the optic chiasma and to the depth of the third ventricle, using a pair of very fine iridectomy scissors. The stalk was then detached, placed in oxygenated Krebs at 37°C and incubated for approximately 20 min. Four median eminence stalks were used for each experiment.

2) The modified perspex tissue holder (see above) was used, and the tissues superfused with Krebs solution at a flow rate of approximately 150µl/min.

3) At the end of the experiment the tissues were removed from the chambers and homogenized in 100µl of 0.1M HCl. 30µl was used for protein estimation and the rest used to estimate the DA and DOPAC content of the tissues, as above, for the DA/DOPAC assay.

C) Retinal tissue: Using the same basic procedure as detailed for striatal tissue, the following modifications were made for experiments with retinal tissue:

1) After killing the rats both the eyes were removed and placed in ice cold Krebs solution. The eye ball was pierced and cut in half,

using fine iridectomy scissors, just behind the cornea. The half containing the retina was held with a fine pair of forceps, turned inside out, and the retina gently teased off. The retina was pre-incubated in Krebs solution at 37°C for 10 min before superfusion. Both the retina from each animal were used for one experiment.

2) At the end of the experiment, the tissue was removed, homogenized, centrifuged, and the supernatant used directly for DA and DOPAC estimation without dilution.

3) The flow rate of the Krebs solution used was approximately 150µl/min.

4) During attempts to induce DA release with light stimulation, the dissection procedure was carried out with greatly reduced background light. The superfusion chambers were enclosed in a black cardboard box, with a window which was opened only during the light stimulation periods. A Xenon tube stroboscope placed 45 cm from the superfusion chambers, flashing at 2-3Hz for 2 min, was used as the light stimulus.

D) Cockroach salivary glands:

1) Both adults and nymphs of *Nauphoeta cinerea* (Olivier) were used (University of Edinburgh, Veterinary School).

2) After excising the legs, wings and antennae, the cockroaches were pinned (through the head and abdomen) onto a pad of silicone gel, under some ice cold Ringers solution. By making cuts along both sides of the body, the dorsal exoskeleton (tergum) was removed (from 3-4 segments of abdomen and thorax) and the gastrointestinal tract pinned to one side of the body. The salivary glands, visualized under the dissecting microscope, were then carefully lifted by holding the

salivary reservoirs, detached from the gut, dissected free and placed in Ringers solution for about 20 min (room temperature).

3) Four glands were used for each experiment, placed in the modified tissue holder (see chamber modification for median eminence tissues) and superfused with oxygenated Ringers solution at room temperature, at a flow rate of 150µl/min. The rest of the superfusion and stimulation procedures were the same as those used for striatal tissues.

4) At the end of the experiments, the tissues were removed and homogenized and centrifuged as described previously, and the supernatant used directly for DA estimation.

Conditions of superfusate analysis

All superfusate samples were collected on ice, into Eppendorf tubes (1.5 ml capacity) at 2 min intervals, using a fraction collector. 7µl of a protective solution containing 1M HCl, 1mM EDTA, 1µM Ascorbate and 2 µg/ml of an internal standard was added to each tube before the onset of collection. The internal standards used were N-acetyl dopamine (nADA) or dihydroxybenzyl amine (DHBA). During experiments with cockroach salivary glands, only DHBA was used as the internal standard.

Recovery of the internal standard was calculated by measuring the flow rate and then working back from the peak height of the authentic standards (injected at the start and end of each experiment) and the dilution factor to give an expected peak height for the internal standard. Only results with a greater than 90% recovery were included, corrections for recovery were not made. Superfusate samples were kept at 0°C, and were always analysed on the same day.

2.5 Other methods

Kainic acid (KA) and 6-hydroxydopamine (6-OHDA) lesions

Rats (200 g male, albino Wistars) were anaesthetized with Equithesin (1 ml injected i.p., approximate duration 3-4 h) and placed in a stereotaxic frame.

2µg of KA (Sigma) in 0.2µl of saline (pH adjusted to 7.4) was unilaterally injected into the striatum. The injection was made over 4 min from a Hamilton syringe (1µl), attached to a 30-gauge needle, the needle was left in place for a further 5 min to prevent reflux. The injection co-ordinates were as follows:

1.0 mm anterior to bregma
2.8 mm lateral to bregma
5.0 mm ventral from brain surface

After pretreatment of the animals with pargyline (50 mg/kg) and desmethylimipramine (25 mg/kg, Geigy Pharmaceuticals) for 30 min, using the same procedure as above, 8µg of 6-OHDA (containing 0.5 mg/ml ascorbic acid) was injected into the lateral hypothalamic area in the region of the Medial Fore-brain Bundle. The co-ordinates for the injection site were:

3.6 mm posterior to bregma
1.0 mm lateral to bregma
8.5 mm ventral from the skull surface

Behavioural assessment of the lesions

Seven to 14 days after the operation, the animals were challenged with apomorphine (MacFarlane Smith Ltd.) to determine turning response to the drug. This was done by placing the rats in a hemispherical plastic bowl, 18 in. in diameter and attaching them to a spring by a length of bandage. The spring was attached to automatic counters

(rat rotometers, see Ungerstedt and Arbuthnott, 1970), counting the number of revolutions made by the rat, in each direction, over a fixed period of time.

The 6-OHDA lesioned animals were given 0.3 mg/kg apomorphine (dissolved in saline + 10μM ascorbic acid) i.p. A response of 200 or more turns, contralateral to the lesioned side, in 45 min was taken to be indicative of a successful lesion.

KA lesioned animals were given 2 mg/kg apomorphine and 50 or more turns, ipsilateral to the lesioned side in 30 min was suggestive of a successful lesion.

A 2 to 7-day rest period was allowed before the animals were used for superfusion experiments after this behavioural assessment.

Histology of the KA lesion site

After removing a slice of brain for the superfusion experiment, the rest of the brain was frozen and mounted on a cryostat chuck for sectioning. 20-40μM slices were cut and melted onto glass slides and stained by a modification of the Kluver and Barrera technique to visualize mylenated fibres and cell bodies.

The procedure is listed below:

a) Dehydrated in 70% alcohol for 5 min.
b) Dehydrated in 95% alcohol for 5 min.
c) Stained for 30 min in a 95% alcohol solution containing 1 mg/ml Luxol Fast Blue (BDH Chemicals Ltd.) and 5μl/ml of 10% acetic acid.
d) Rinsed in 95% alcohol to remove excess stain.
e) Rinsed in distilled water.
f) Differentiated by immersion in 0.05% lithium carbonate for 10 sec, followed by immersion in 70% alcohol for 30 sec. The differentiation steps were repeated four or five times until a clear distinction was seen between the blue stained white matter and the colourless grey matter.
g) Washed in distilled water and transferred to a solution containing 0.1% Cresyl Fast Violet (Fluka) in distilled water for 5 min.

h) Rinsed in distilled water.

i) Dehydrated in changes of 70%, 95% and 100% alcohol, 5 min each.

j) Cleared in Xylene for 10 min.

k) Coverslipped using DPX (BDH Chemicals Ltd.)

The slides were then examined under a light microscope.

Collection of portal blood and alumina extraction

Portal blood was collected by the method of Worthington (1964) (as modified by Fink and Jamieson, 1976), spun down for 10 min at 2000 rpm, and the plasma aliquoted (50-100µl samples) into tubes containing 10µl of a 1mM ascorbic acid and 10 ng/ml nADA solution. The tubes were stored at -70°C. For analysis the plasma was thawed, the proteins precipitated by adding 30µl of 1M trichloroacetic acid, and spun at 12,000 rpm for 5 min. 50µl of 1mM sodium thiosulphate was then added to the supernatant followed by approximately 25 mg of acid washed alumina (Bioanalytical Systems). To this was added 500µl of 1M Tris buffer (pH adjusted to 8.6) and the tubes shaken for 15 min in a cold room. After allowing the alumina particles to settle, the supernatant was aspirated off and the alumina washed four times, twice with 0.1mM Tris buffer and twice with distilled water. The alumina was then dried in a vacuum desiccator after which the catecholamines were eluted into 60µl of 0.5M HCl. After shaking for 5 min in the cold room, the tubes were spun for 30 sec, to settle the alumina. The supernatant was then injected into the HPLC-ECD to measure DA, etc.

2.6 Materials

All chemicals used were "Analar" grade, from British Drug House. The radiolabelled DA used was: 1) [^{3}H-7]-Dopamine, New England Nuclear (20-40mCi/mM for experiments described in 3.1);

2) [^{3}H-7,8]-Dopamine, Amersham (30-50Ci/mM for all other experiments).

Unless otherwise stated, all drugs, catecholamines and their metabolites were obtained from the Sigma Chemical Company. The following compounds were generously donated by the parties mentioned:

1. LY5953A, Eli Lilley and Co.
2. Nomifensine, Hoechst Pharmaceuticals.
3. 3-(3-hydroxyphenyl)-N-n-propylpiperidin hydrochloride, (3PPP), Astra Lakemedel AB.

CHAPTER III

Results

3.1 Identity of the DA peak

In order to confirm that the peak eluting at the retention time of authentic DA, from tissue homogenates or superfusate samples, was actually DA several tests were carried out.

(a) U.V. Spectrophotometry

Connecting the outflow of the ECD cell to a spectrophotometer cell, as described in Chapter II, 2.3.1, allowed the simultaneous measurement of the electrochemical signal and the UV absorbance of the compound. This set-up could be used successfully only if large doses of standards were injected. Compounds like tyrosine, tyramine and octopamine (redox potentials greater than +0.8V) could be detected by spectrophotometry but not by ECD (set at a working potential of +0.65V). It was not possible to simultaneously measure catecholamines from tissue homogenates with any degree of accuracy, as spectrophotometry proved to be about four orders of magnitude less sensitive than ECD (see Figure 3.1).

(b) Gas Chromatography with Mass Spectrometry (GCMS)

The supernatants of tissue homogenates were simultaneously analysed by HPLC-ECD and GCMS for DA. The results obtained are in good agreement.

	HPLC-ECD	GCMS
Striatal tissue (n=3)	92.3 ± 6.1 ng/mg protein	90.05 ± 8.2 ng/mg protein
Cockroach salivary glands (n=4)	1.9 ± 0.2 ng/ gland	2.0 ± 0.2 ng/ gland

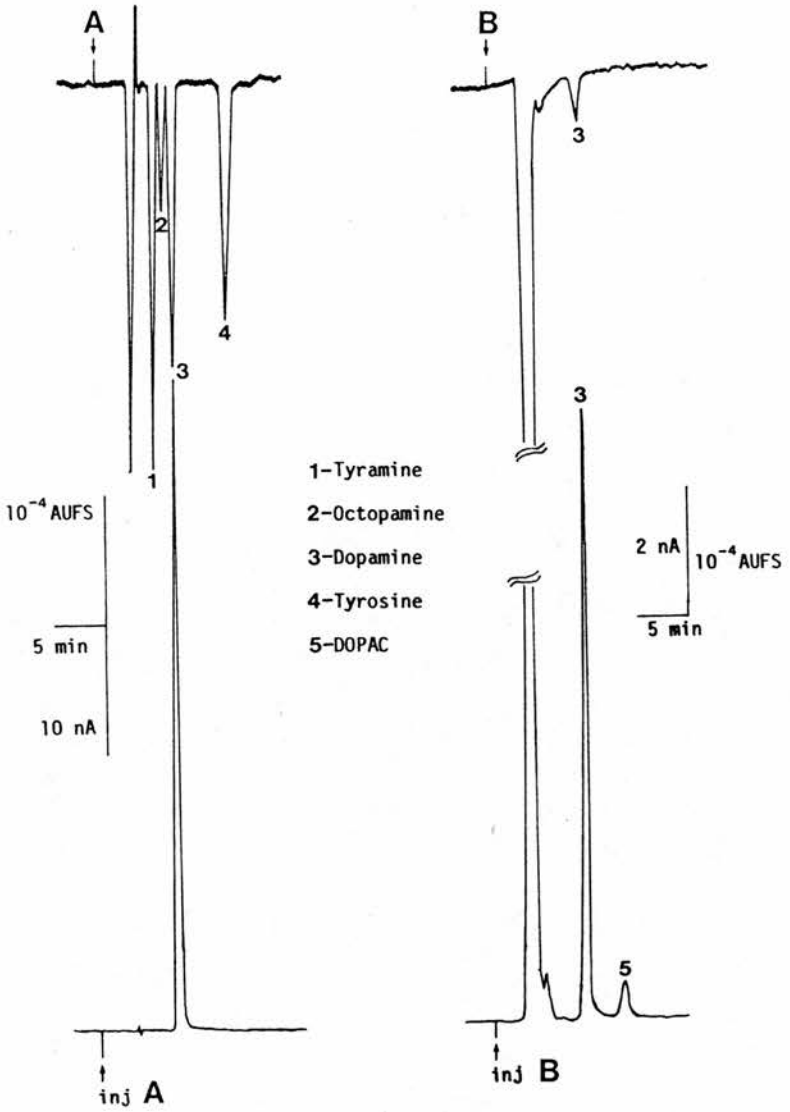


FIGURE 3.1: Simultaneous detection of catecholamines, their precursors and metabolites by:

Top inverted trace = U.V. spectrophotometry at 280nM

Bottom trace = Electrochemical detection at +0.65V.

A: An injection (at point marked by arrow) of standards under standard chromatographic conditions (see page 47 for details).

B: An injection of striatal tissue extract.

(c) Alteration of chromatographic conditions

The supernatant of homogenates of striatal tissue was analysed by HPLC-ECD under different chromatographic conditions (see Figure 2.6, 3.2). The results, tabulated below, show that changes in the chromatographic condition did not alter the observed DA and DOPAC content of the tissues. The retention time of DA and DOPAC in the samples always corresponded to those of authentic DA and DOPAC.

Buffer composition	DA t_r (min)	DA content (ng/mg protein)	DOPAC t_r (min)	DOPAC content (ng/mg protein)
Standard (see page 48)	6.75	94.2 ± 3.8 (n=6)	10.0	4.8 ± 0.4 (n=6)
pH = 3.5, rest as above	7.0	89.4 ± 4.0 (n=4)	12.0	5.2 ± 0.5 (n=4)
5% methanol, rest as above	16.5	86.9 ± 6.1 (n=3)	23.0	-
125 mg sodium octyl sulphonate	12.5	96.8 ± 5.1 (n=4)	10.5	6.1 ± 0.8 (n=4)

(d) Alumina extraction

Striatal slices, superfused with Krebs solution were stimulated once with 25mM K^+ Krebs, as described in Section 2.2. The superfusate sample collected during the stimulation period (i.e. 16 min from the onset of superfusion) was first analysed by HPLC-ECD to determine the DA and DOPAC content. DA and DOPAC were then extracted from the rest of the supernatant using acid washed alumina (see page 62). The eluent of the alumina extracts was injected into the HPLC-ECD, and the total DA and DOPAC content of the superfusate determined after correction for the recovery. The DA and DOPAC content of the superfusate by direct analysis or after alumina extraction are listed below.

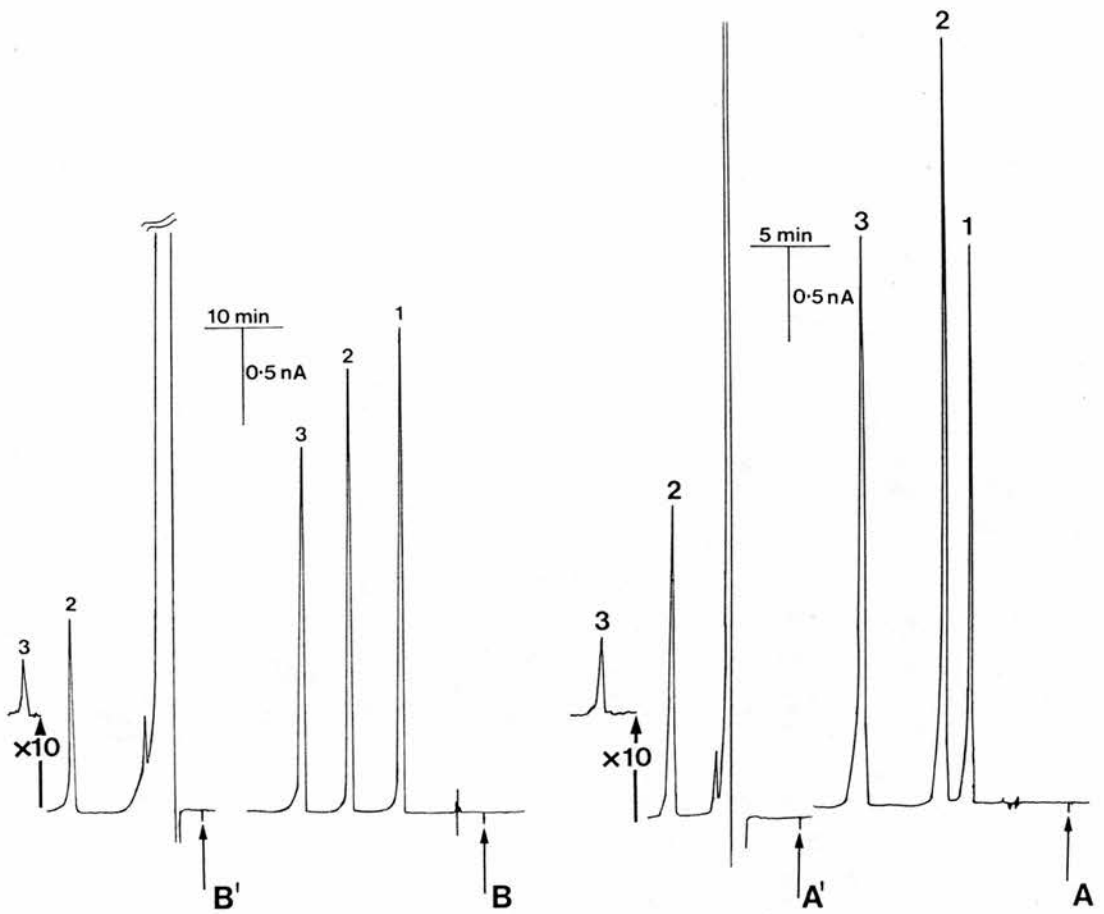


FIGURE 3.2: Comparison of the retention times of authentic standards and striatal tissue extracts for dopamine and DOPAC (see also Figure 2.6).

1 = dihydroxybenzylamine
2 = dopamine
3 = dihydroxyphenylacetic acid (DOPAC)

At A, A' standard chromatographic conditions were used (see page 47).

A = standards - 1 ng of each.
A' = striatal extract, diluted 1 in 10 after homogenization in 1 ml, using approximately 15 mg wet weight of tissue, 20µl injection volume (see page 56).

At B, B' the methanol content of the buffer was reduced to 5%.

B = standards, as above.
B' = striatal extract, as above.

ng/mg protein	Direct analysis	Alumina extract (corrected for recovery)
DA	4.05 ± 0.43	3.72 ± 0.41
DOPAC	0.89 ± 0.08	0.91 ± 0.09

No significant difference was found between the two sets of values.

(e) Chemical composition of released tritium

Striatal tissue, preincubated with ^{3}H-DA (20µl of [^{3}H-7]-dopamine) was stimulated once with a 2 min pulse of 35mM K^+. The supernatant fractions were then analysed as follows:

1. DA overflow by HPLC-ECD, in 20µl of the superfusate.
2. Total ^{3}H overflow. Expressed as a fractional rate constant X100, i.e. [release ^{3}H (cpm) ÷ total ^{3}H (cpm) left in tissue & supernatant at the start of the respective 2 min collection] X100.
3. The HPLC eluent from 1. was collected at 1 min intervals and the radioactivity determined in each sample.

The results are shown in Figure 3.3. 1) A 2 min pulse of 35mM K^+ greatly enhanced the overflow of DA as measured by HPLC-ECD and as total ^{3}H overflow, concomittantly. 2) The radioactivity profile of the HPLC separation shows that during the stimulation period, most of the ^{3}H released coelutes with the DA peak. Expressing the amount of radioactivity under the DA peaks as a percentage of the total radioactivity in the sample, showed that basal overflow (1.4 ± 0.1% fractional overflow) consisted of 15 ± 3% ^{3}H-DA; during stimulation with 35mM K^+, the 13 ± 1% fractional overflow of ^{3}H consisted of 72 ± 8% ^{3}H-DA. Changes in the overflow of ^{3}H-DOPAC were not analysed as the ^{3}H was attached to the side chain of the dihydroxy-

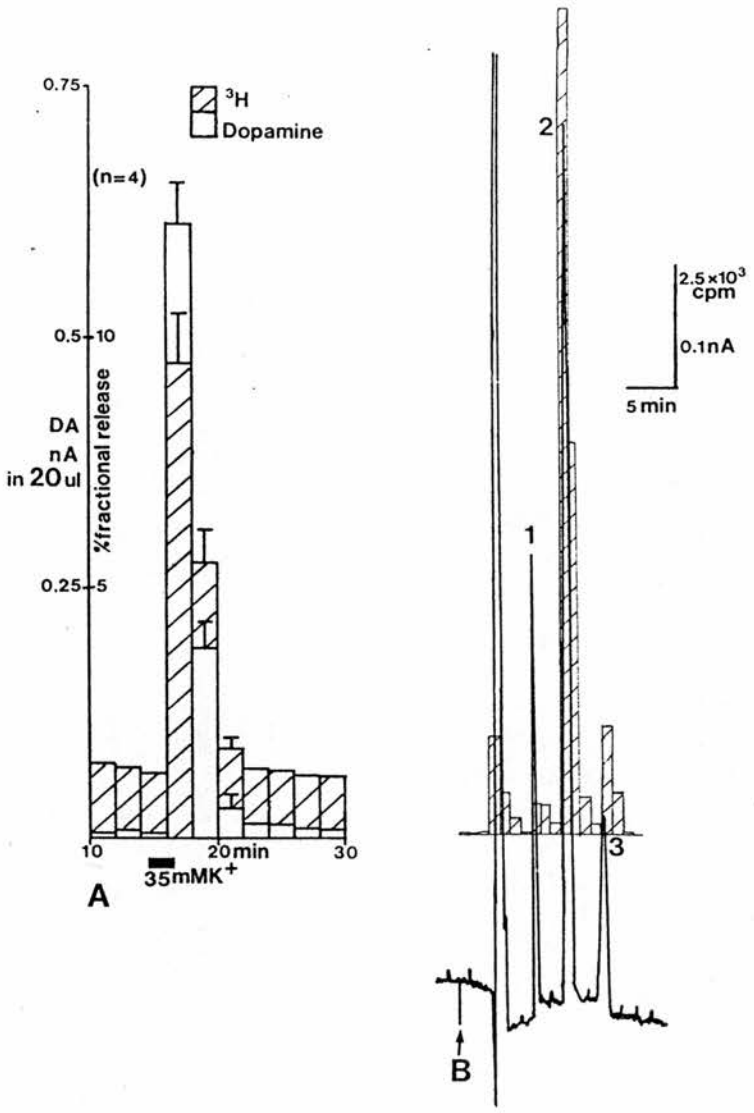


FIGURE 3.3: Striatal tissue preincubated with ^{3}H-DA was stimulated once with a 2 min pulse of 35mM K^+. The superfusate was analysed for its DA content by HPLC-ECD and its ^{3}H content by liquid scintillation counting.

A = comparison of the DA overflow (expressed as nA in 20µl of superfusate) and total ^{3}H overflow (expressed as % fractional release). Vertical bars show s.e.m. (only sem >15% are shown). The overflow of both DA and ^{3}H is simultaneously and rapidly increased by 35mM K^+ Krebs.

B = Radioactive (^{3}H) profile of an HPLC separation of the superfusate; sample taken during superfusion with 35mM K^+

1 = Dihydroxybenzylamine - internal standard
2 = Dopamine
3 = Dihydroxyphenylacetic acid (DOPAC)

More than 70% of the ^{3}H present in the sample co-elutes with dopamine.

phenyl ring and so may have been lost during deamination with MAO (Starke *et al.*, 1980), as indicated by the ^3H associated with the solvent front.

3.2 K^+ induced overflow of DA from superfused striatal slices

Experiments carried out to investigate the K^+ induced overflow of DA/DOPAC, and its alteration by physiological or pharmacological manipulation are described in this section.

Unless otherwise stated, striatal slices (approximately 0.3 x 0.3 x 1 mm) superfused with oxygenated Krebs at 37°C, at a flow rate of approximately 350µl/min were stimulated twice with 25mM K^+ at 16 and 56 min from the onset of superfusion. Drugs were added 30 min after onset of superfusion (i.e. before S_2) to allow a comparison between drug induced effects at S_2 and the internal control S_1. All results were calculated according to the following equation (as demonstrated in Appendix I):

$$\text{ng/mg protein/2 min} = \frac{\text{peak height}}{\text{peak height of standards}} \times \text{amount of standards} \times \frac{\text{flow rate/2 min}}{\text{injection volume}} \times \frac{1}{\text{proteins}}$$

(a) 25mM K^+ induced overflow of DA and DOPAC. Control

Basal overflow: during incubation with Krebs solution, the initial basal overflow of DA was found to be 0.08 ± 0.02 (range 0.29 to >0.02) ng/mg protein/2 min (mean ± s.e.m., n=8). The initial basal overflow of DOPAC was found to be much higher, i.e. 0.65 ± 0.05 (range 1.05 to 0.42) ng/mg protein/2 min. The basal release of DOPAC 50 min after start of superfusion, i.e. before the second stimulation, S_2, was found to be significantly higher, i.e. 0.96 ± 0.08 ng/mg protein/2 min

($p < 0.05$, two-tailed, paired student "t" test, see Figure 3.5), but the DA basal overflow did not differ from that at S_1.

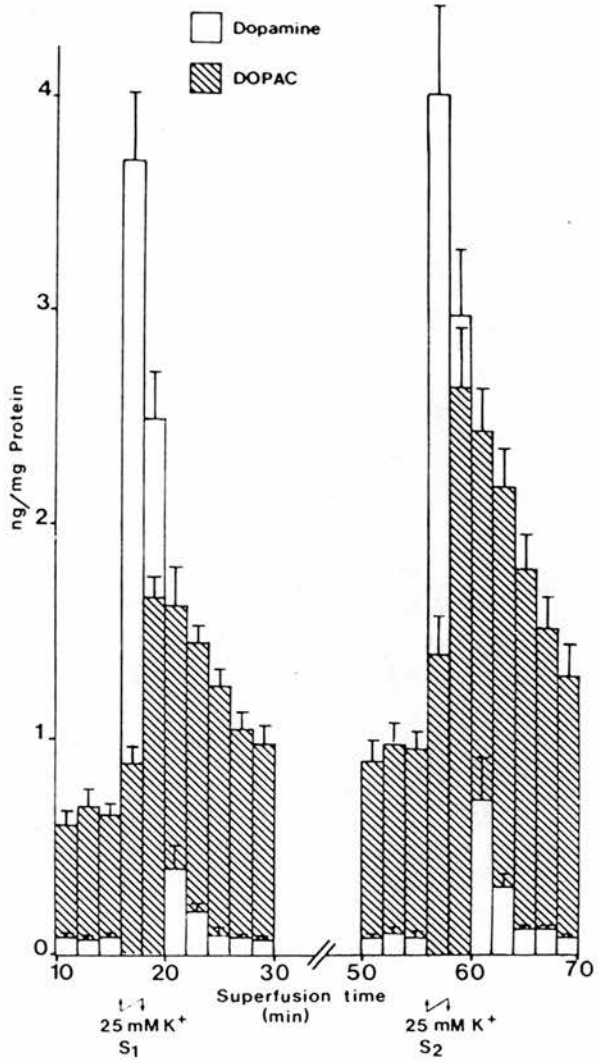
Evoked overflow: as shown in Figure 3.4, the evoked overflow of DA, induced by a 2 min pulse of high K^+ Krebs, was complete (>95%) within 6 min of the onset of stimulation, i.e. 3 sample times. The K^+ induced overflow of DA was, therefore, estimated as the total increase (above basal overflow) in DA overflow during these 6 min, and found to be 6.3 ± 0.4 (range 4.34 to 7.85) ng/mg protein during the first stimulation (S_1). A second 2 min pulse of 25mM K^+, S_2, consistently induced an approximately 20% higher overflow of DA, i.e. 7.7 ± 0.6 (range 10.4 to 5.8) ng/mg protein.

DOPAC overflow after K^+ stimulation showed a slower rate of increase and decline. The increase in DOPAC, during the 6 min after the onset of stimulation, was used to compare changes in the evoked overflow of DOPAC. Hence the increase in DOPAC overflow during 25mM K^+ stimulation was taken as 2.24 ± 0.14 (range 3.06 to 1.8) ng/mg protein during S_1 and 3.69 ± 0.28 (range 5.02 to 2.62) ng/mg protein at S_2. The difference was significant at the $p < 0.05$ level by a two-tailed, paired student "t" test (see Figure 3.5).

(b) Ca^{++} dependence

Substituting 0.1mM EGTA for calcium chloride in the Krebs solution during the second stimulation, S_2, with 25mM K^+ Krebs, resulted in the complete inhibition of the stimulation evoked increase in the overflow of DA and DOPAC as shown in Figure 3.6.

FIGURE 3.4: Striatal slices superfused with Krebs solution were stimulated twice, at S_1 and S_2, with a two-minute pulse of 25mM K^+. Two-minute fractions of the superfusate were collected and analysed for their DA and DOPAC content by HPLC-ECD. The results are shown below and expressed as ng DA or DOPAC/mg protein in each fraction (vertical bars show s.e.m., n=8). A summary of the results is shown overleaf.



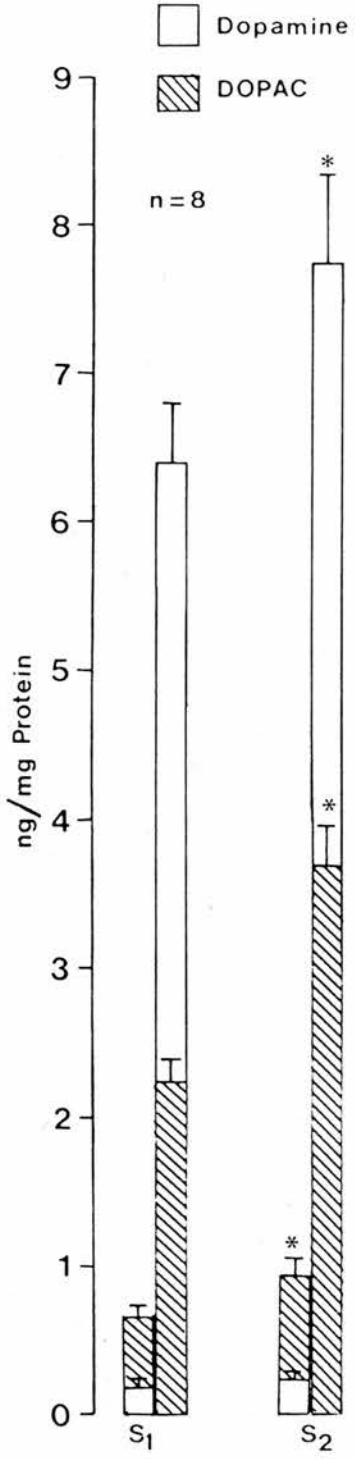


FIGURE 3.5: Summary of the results of two (S_1 and S_2) 2 min pulses of 25mM K^+ Krebs, shown in detail in Figure 3.4.

Each pair of histograms represent the basal and the evoked (25mM K^+) overflow of DA and DOPAC in that order (vertical bars = s.e.m.). The basal overflow of DOPAC and the evoked overflow of both DA and DOPAC were significantly higher at S_2 when compared to the internal control S_1 (* = $p < 0.05$, two-tailed, paired Student 't' test).

For this and subsequent diagrams the basal overflow was calculated as the average overflow of DA/DOPA found during the 6 min before the onset of stimulation, i.e. between 10-16 min for S_1 and 50-56 min for S_2 from the onset of superfusion. Hence, basal overflow measurements are shown as ng DA or DOPAC/mg protein/ 2 min.

The evoked overflow of DA/DOPAC was taken as (see text) the total amount of DA/ DOPAC during the 6 min after the onset of stimulation minus the basal overflow and shown as ng DA or DOPAC/mg protein (see Figure 3.4).

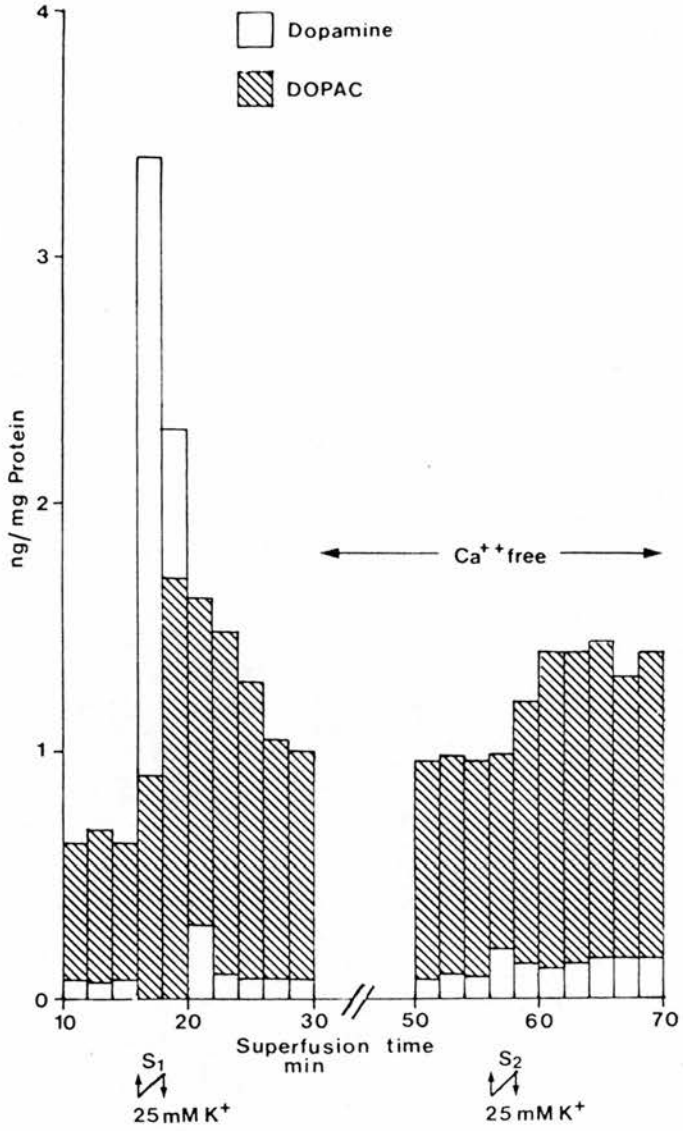


FIGURE 3.6: Histograms showing the DA and DOPAC content of the superfusate from striatal tissue, superfused with normal Krebs solution and stimulated with a 2 min pulse of 25mM K^+ Krebs at S_1 and then superfused with Ca^{++} free Krebs (starting 30 min after the onset of superfusion) and again stimulated with 25mM K^+ (Ca^{++} free) Krebs at S_2 (n=3). Omission of Ca^{++} from the Krebs and 25mM K^+ Krebs solution abolished the 25mM K^+ induced increase of DA and DOPAC overflow. A small ongoing increase in the basal overflow of both DA and DOPAC was noted in the absence of Ca^{++}.

(c) Potassium concentration

Striatal slices (approximately 16 mg wet weight) superfused with Krebs solution (325µl/min), were stimulated once each with 15, 25, 35 or 50mM K^+ for 2 min. The results, calculated as above, are shown in Figure 3.7. The large increase in DA overflow with increasing K^+ concentration is accompanied by a smaller increase in DOPAC overflow.

(d) Influence of muscarinic agents on DA and DOPAC overflow

i) Indirectly acting muscarinic agonists:

In order to evaluate the influence of cholinergic agents on DA overflow, acetylcholine esterase inhibitors were used to see the effect of increased endogenous ACh release on the overflow of DA.

Physostigmine added 30 min after the onset of superfusion resulted in a dose dependent increase in the overflow of DA evoked by a 2 min pulse of 25mM K^+ at S_2. Using the first stimulation, S_1, as an internal control, the results were expressed as the ratio S_2/S_1. The control, S_2/S_1, for DA was found to be 1.20 ± 0.04 (n=10, range 1.49 to 0.94) and 1.74 ± 0.12 for DOPAC (n=10, range 2.58 to 1.31). Addition of 1µM physostigmine elevated the evoked overflow of both DA and DOPAC as indicated by the increased S_2/S_1 ratios for DA, 2.19 ± 0.17 (n=6, range 1.75 to 2.86) and for DOPAC, 2.88 ± 0.10 (n=6, range 2.68 to 3.22). The dose-response relationship for physostigmine is shown in Figure 3.8.

Neostigmine (1µM) similarly increased the S_2/S_1 ratios for the evoked overflow DA to 2.23 ± 0.09 (n=8, range 1.91 to 2.69) and DOPAC to 2.38 ± 0.10 (n=8, range 2.14 to 3.05). The addition of tubocurarine (1µM) or gallamine (1µM) along with neostigmine (1µM)

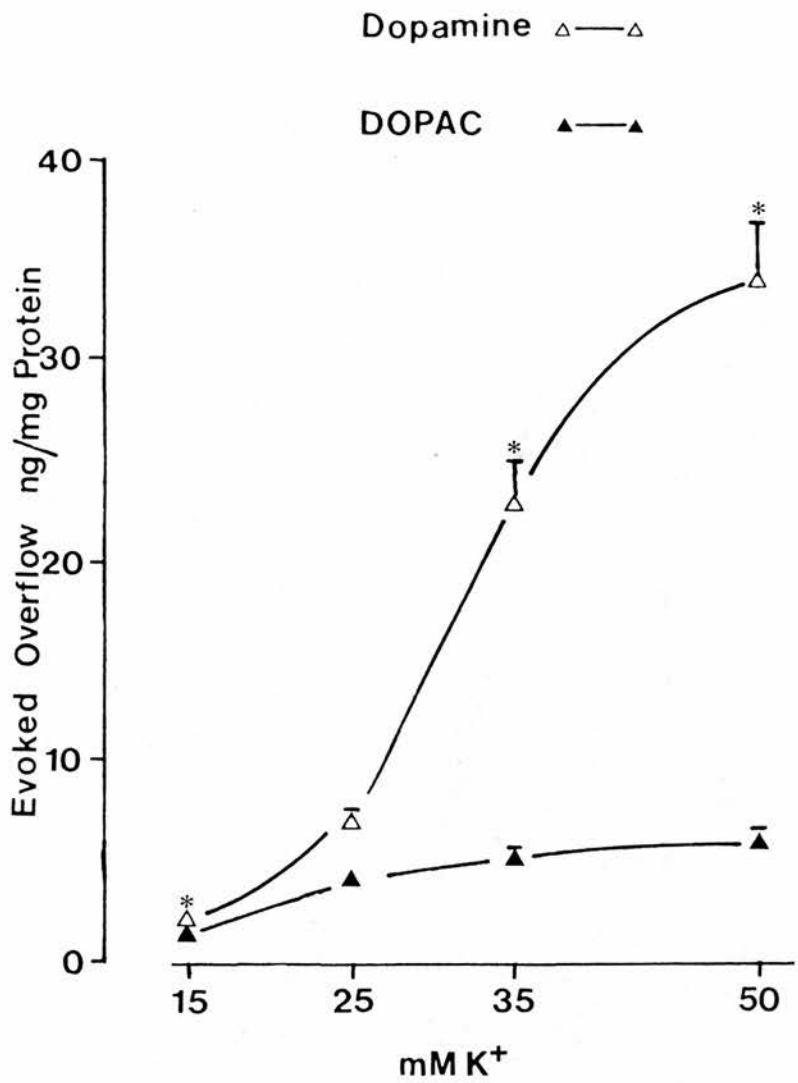


FIGURE 3.7: Striatal slices superfused with Krebs solution were stimulated once with a two-minute pulse of 15, 25, 35 or 50mM K^+. The evoked overflow of DA and DOPAC (calculated as in Figure 3.5) is plotted against the K^+ ion concentration (vertical bars = s.e.m.; s.e.m.'s smaller than figure notations are now shown. n=4).

The very large difference in the increase of DA overflow between 15 and 50mM K^+ stimulation was accompanied by only a small difference in the DOPAC overflow.

P >0.05, Student 't' test, two-tailed, show differences from the overflow of DA/DOPAC seen with 25mM K^+ Krebs.

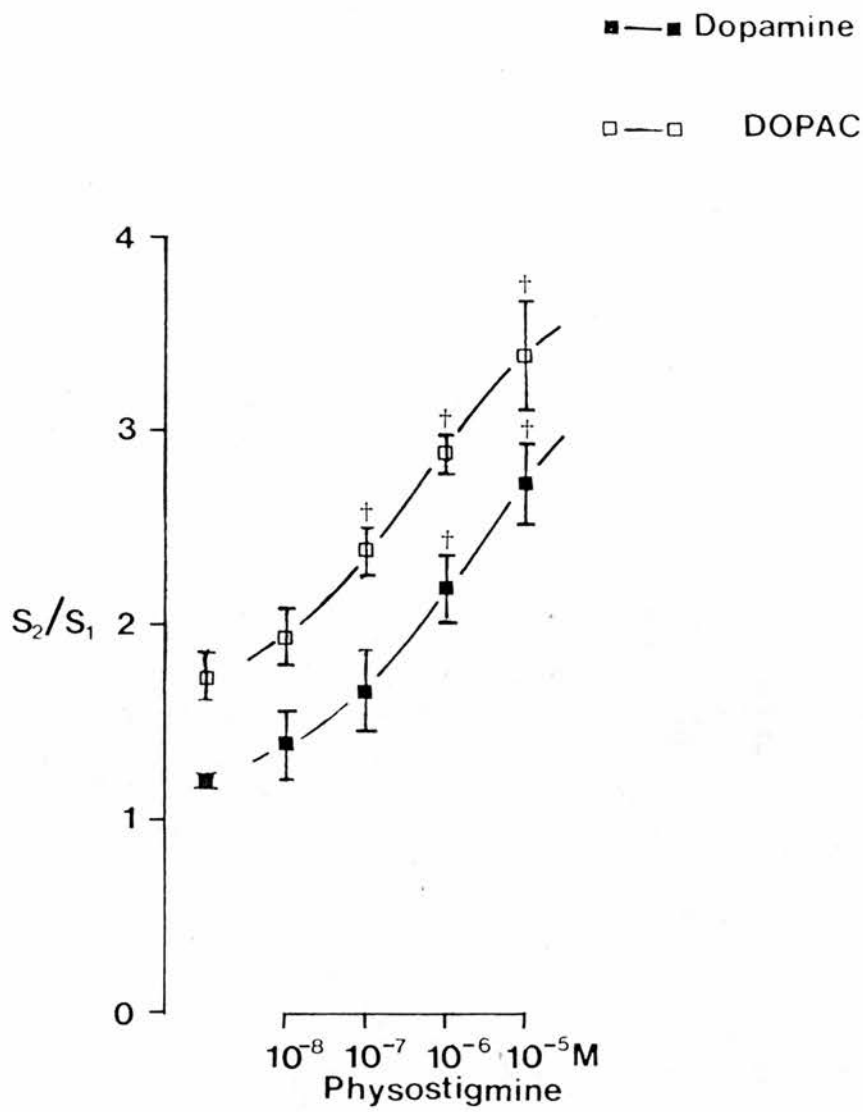


FIGURE 3.8: The dose/response relation for physiostigmine. Striatal slices superfused with Krebs solution were stimulated with 25mM K^+ at 16 min from the onset of superfusion as the internal control, S_1. At 30 min, physiostigmine (0.01 to 10μM) was added to the superfusing Krebs solution and the slices stimulated again with 25mM K^+ at 56 min for the test S_2.

The ratio of the test stimulation S_2 to the internal control S_1 for the evoked overflow of DA and DOPAC is shown above (vertical bars = s.e.m., n=3-6).

Physiostigmine dose dependently enhanced the 25mM K^+ evoked overflow of DA, as indicated by the increase in the S_2/S_1 ratios for DA. Similarly, DOPAC overflow was also enhanced by physiostigmine, indicating an increased synthesis of DA (see later). The basal overflow of DA and DOPAC was not affected by physiostigmine. † - $p < 0.05$, Wilcoxon rank test, indicates values significantly different from control.

did not alter the response of neostigmine (see Figure 3.9). Atropine (1μM) added with neostigmine, however, abolished the facilitatory effect of neostigmine on DA and DOPAC overflow. Atropine (1μM) added alone reduced DA and DOPAC overflow, the S_2/S_1 ratios being 0.85 ± 0.10 (n=4, range 1.14 to 0.65) for DA and 1.45 ± 0.15 (n=4, range 1.95 to 1.19) for DOPAC.

ii) Directly acting muscarinic agonist:

Addition of ACh (100μM) to the Krebs solution before S_2 similarly increased the S_2/S_1 ratios for both DA and DOPAC to 1.68 ± 0.16 (n=4, range 1.38 to 2.07) and 1.97 ± 0.07 (n=4, range 1.82 to 2.13) respectively. The effect of ACh was further increased at 1mM and could be abolished by the simultaneous addition of atropine (1μM) (see Figure 3.10).

None of the above mentioned drugs altered the basal overflow of either DA or DOPAC at any stage.

In an attempt to reduce ACh release, hemicholinium-3 (HC-3) (5μM) was added to striatal slices from the start of the experiment. However, these experiments had to be abandoned as despite an increased DA overflow on stimulation with 25mM K^+, the metabolism of DA appeared to be drastically altered. Several unidentified peaks appeared replacing the DOPAC peak, making measurement of DA overflow difficult and casting doubt on the relevance of changes in DA overflow (see Figure 3.11).

(e) KA lesion

The 25mM K^+ evoked overflow of DA and DOPAC from KA lesioned rats, from both the lesioned and unlesioned sides (superfused separately from each unilaterally lesioned animal) did not appear to differ from

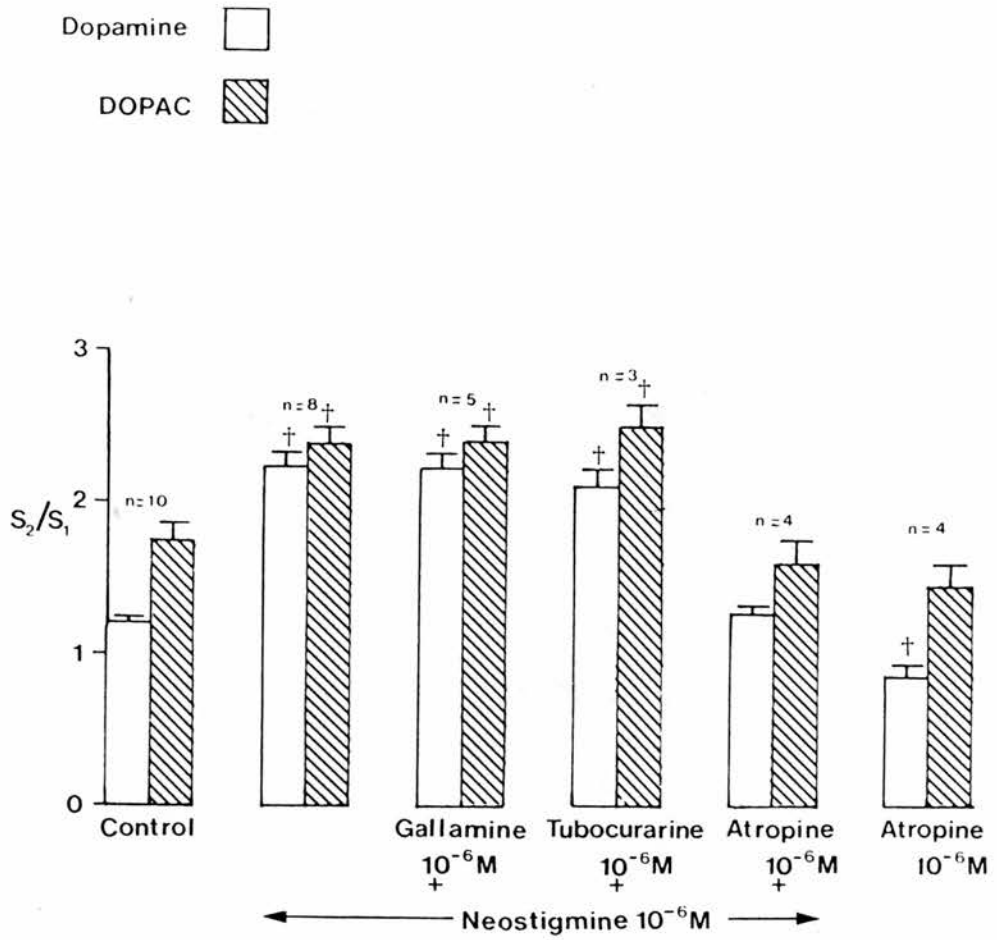


FIGURE 3.9: The effect of cholinergic antagonists on the facilitation of DA and DOPAC evoked overflow induced by neostigmine.

As in Figure 3.8, neostigmine (1μM) added 30 min after the onset of superfusion, enhanced the 25mM K^+ evoked overflow of DA and DOPAC, indicated by the increases in the S_2/S_1 ratios.

The nicotinic antagonists, gallamine (1μM) or tubocurarine (1μM) added with the neostigmine, failed to alter the response of neostigmine. The muscarinic agonist, atropine (1μM) added with neostigmine completely abolished its effects.

Atropine (1μM) added alone reduced slightly the overflow of DA. This indicates that a stimulation of muscarinic presynaptic receptors facilitates the release and synthesis of DA (see later). † - values significantly different from control, $p < 0.05$, Wilcoxon Rank test (as with all diagrams, vertical bars = s.e.m.).

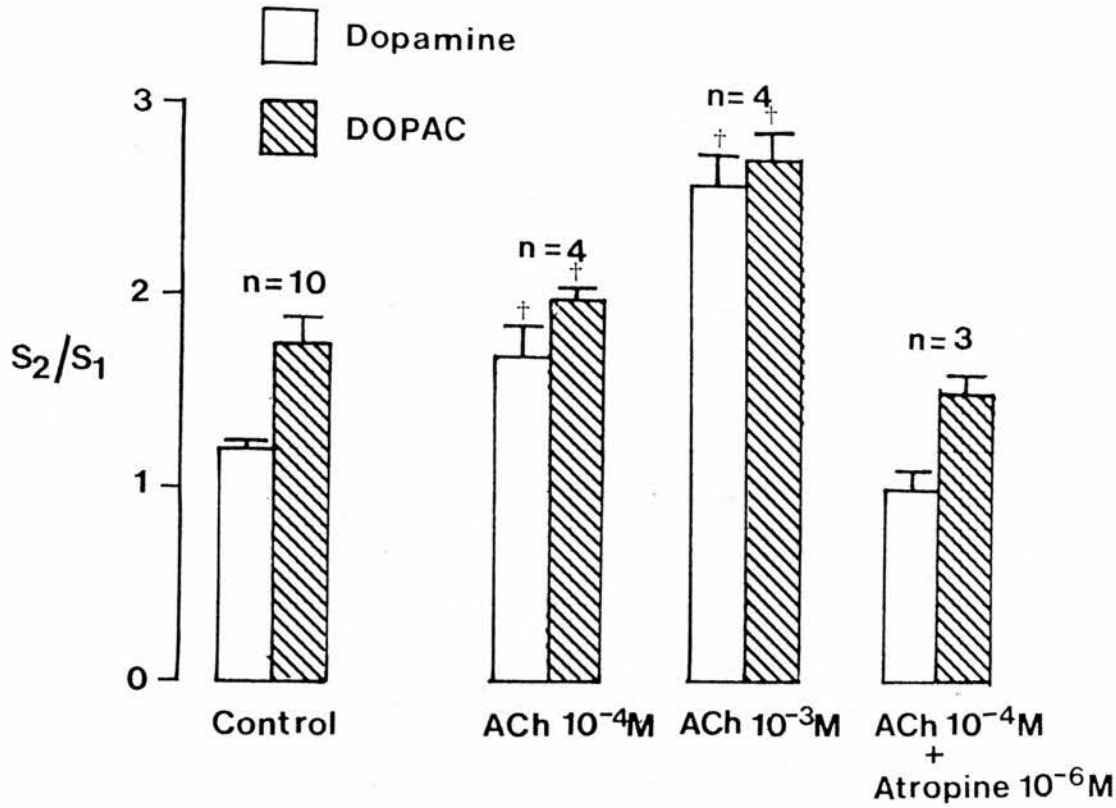


FIGURE 3.10: Facilitation of the evoked overflow of DA and DOPAC by acetylcholine, and its inhibition by atropine.

As in Figure 3.8, ACh (0.1mM) added to the Krebs solution at 30 min, enhanced the 25mM K^+ evoked overflow of DA and DOPAC at S_2. Hence the S_2/S_1 ratios were significantly increased. ACh (1mM) further enhanced the overflow of DA and DOPAC.

The facilitatory effects of ACh (0.1mM) on the evoked overflow of DA and DOPAC were abolished by the simultaneous addition of atropine (1μM), again indicative of a muscarinic receptor mediated effect.

(†, $p < 0.05$, Wilcoxon Rank test)

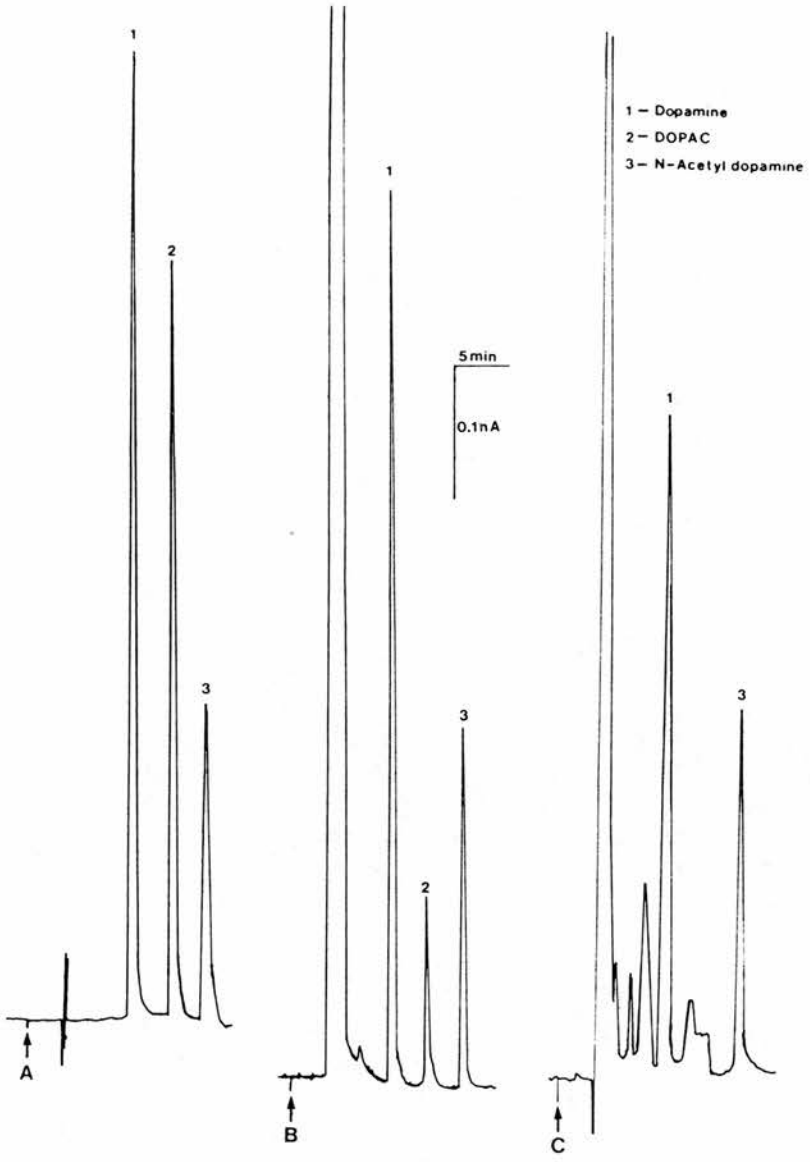


FIGURE 3.11: Comparison of HPLC-ECD traces obtained during a 25mM K^+ induced release from a control experiment, and in the presence of hemicholinium-3.

A = Standards, 0.2 ng, N-acetyldopamine was used as the internal standard.

B = A 20µl injection of the superfusate of a control experiment, collected during a 25mM K^+ stimulation at 56 min (i.e. S_2).

C = A 20µl injection of the superfusate during an experiment conducted in the presence of hemicholinium-3 (present from the start of superfusion). The superfusate was collected during a 25mM K^+ stimulation at 56 min (i.e. S_2).

controls (see Figure 3.12). The lesion site was confirmed by behavioural tests (see page 60) and histologically (see Figure 3.13). Although the lesions were extensive this did not appear to interfere with the response to neostigmine (1μM) added 20 min before S_2 (see Figure 3.12).

(f) Effect of uptake inhibitors on 25mM K^+ induced overflow of DA

Addition of nomifensine (1μM) before S_2 did not alter either the basal overflow or the 25mM K^+ evoked overflow of DA or DOPAC, as shown below.

		Basal overflow ng/mg protein/ 2 min		Evoked overflow ng/mg protein	
		DA	DOPAC	DA	DOPAC
Control (n=8)	S_1	0.08 ± 0.02	0.65 ± 0.05	6.3 ± 0.4	2.24 ± 0.14
	S_2	0.09 ± 0.02	0.96 ± 0.08	7.7 ± 0.6	3.69 ± 0.28
Nomifensine (1μM) added 26 min before S_2 (n=4)	S_1	0.08 ± 0.02	0.59 ± 0.06	5.8 ± 0.6	2.18 ± 0.20
	S_2	0.10 ± 0.04	0.90 ± 0.08	8.2 ± 0.9	3.80 ± 0.40

(g) Comparison between DA and ^3H overflow

i) Simultaneous DA and ^3H overflow induced by 25mM K^+:

Striatal slices were preincubated with ^3H-DA (4μl of [^3H-7,8]-dopamine 47Ci/mM) before the onset of superfusion. The slices were stimulated twice (S_1 and S_2) with 25mM K^+, as above. The superfusate was analysed as follows: 1) a 20μl sample was injected into the HPLC-ECD to measure DA and DOPAC; 2) 500μl of the superfusate was added to 8 ml of scintillant and its ^3H content determined by liquid scintillation counting.

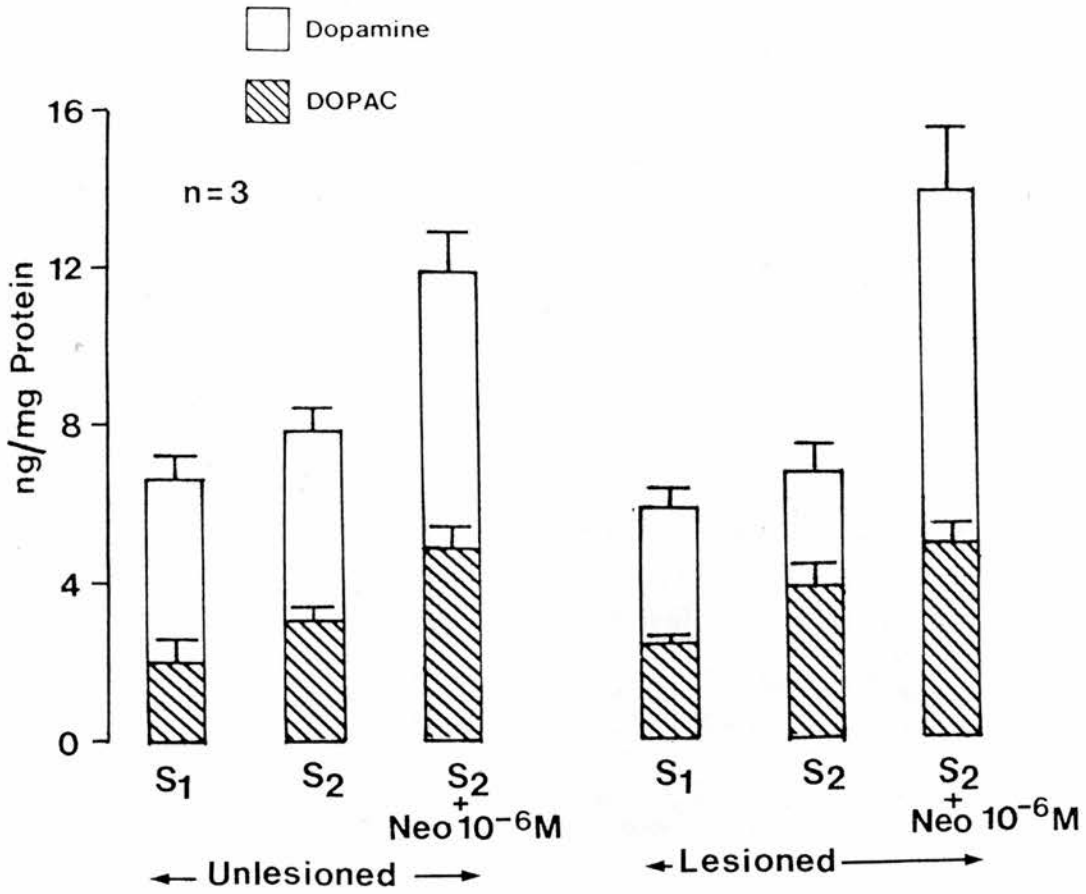


FIGURE 3.12: Unilaterally, kainic acid lesioned animals, were tested behaviourally and histologically (see Figure 3.13) for successful lesions. The lesioned and unlesioned striata from each animal were superfused separately with Krebs solution, and stimulated twice with 25mM K^+. Neither the basal (not shown) nor the evoked overflow of DA or DOPAC (above) at S_1 or S_2 differed from control animals (see Figure 3.5), from both the lesioned and the unlesioned sides.

The addition of neostigmine (1μM) 30 min after the onset of superfusion greatly enhanced the overflow of both DA and DOPAC at S_2, from both the lesioned and the unlesioned sides.

(vertical bars = s.e.m.)

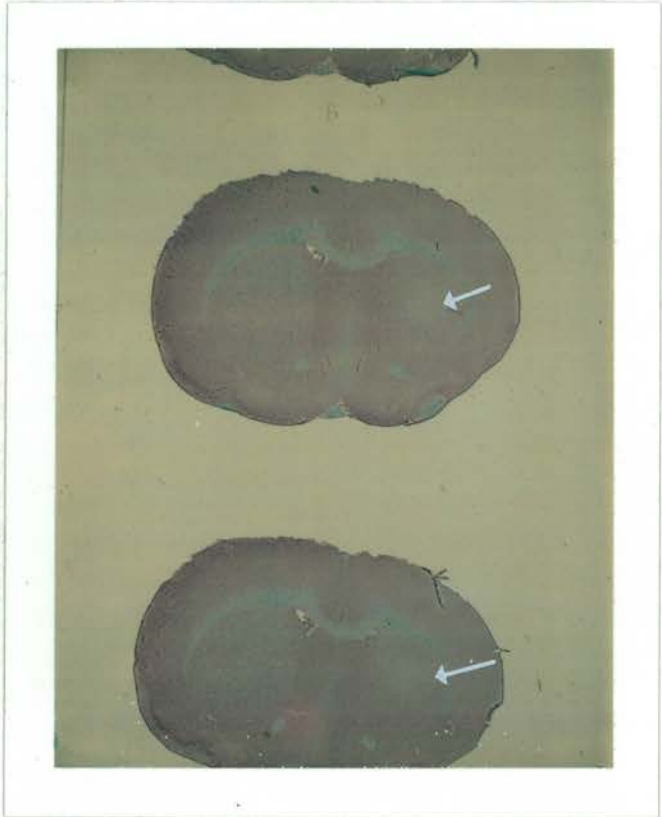


FIGURE 3.13: Histology of the kainic acid lesion site. After cutting the slice of brain required for the superfusion experiments, the remainder of the brain was frozen, sectioned and stained (as on page 61) and examined under a light microscope. The lesion site was usually visible with the naked eye, as the low power photograph above shows.

The basal and evoked overflow of DA did not differ from experiments carried out in the absence of ^{3}H-DA (see Figure 3.14). The evoked overflow of DA was 6.6 ± 0.5 (range 8.25 to 4.2) ng/mg protein (n=8) at S_1 and 8.5 ± 1.2 (range 10.64 to 4.47) ng/mg protein at S_2, giving an S_2/S_1 ratio of 1.29. Although the basal overflow of DOPAC did not differ from experiments carried out in the absence of ^{3}H, there appeared to be a greater evoked overflow of DOPAC at S_1, i.e. 3.38 ± 0.35 (range 5.46 to 2.11) ng/mg protein, but a near control level at S_2, i.e. 3.61 ± 0.71 (range 5.88 to 2.11) ng/mg protein reducing the S_2/S_1 ratio for DOPAC to 1.07 (cf. 1.74, page 71 and Figure 3.5).

The evoked overflow of ^{3}H (expressed as fractional release X 100, as before) at S_1 was found to be 17.0 ± 1.3% (n=8, range 23.9 to 11.4%) and 14.7 ± 2.1% (n=4, range 19.9 to 8.41%) at S_2, giving an S_2/S_1 ratio of only 0.87 (see Figure 3.14). Hence, while the overflow of endogenous DA was higher at S_2, the overflow of ^{3}H was lower at S_2, compared to S_1.

ii) The influence of neostigmine on the ^{3}H and DA/DOPAC overflow:

Addition of neostigmine (1μM) 26 min before S_2, markedly increased the overflow of both DA (14.28 ± 0.45, range 14.07 to 15.65 ng/mg protein) and DOPAC (8.63 ± 0.6, range 7.09 to 10.36 ng/mg protein) giving S_2/S_1 ratios of 2.16 for DA and 2.39 for DOPAC. There was, however, a more modest increase in the overflow of ^{3}H to 21.6 ± 1.6% (n=4, range 17.28 to 25.37) which was not statistically different from control, giving a S_2/S_1 ratio of 1.27 (see Figure 3.14).

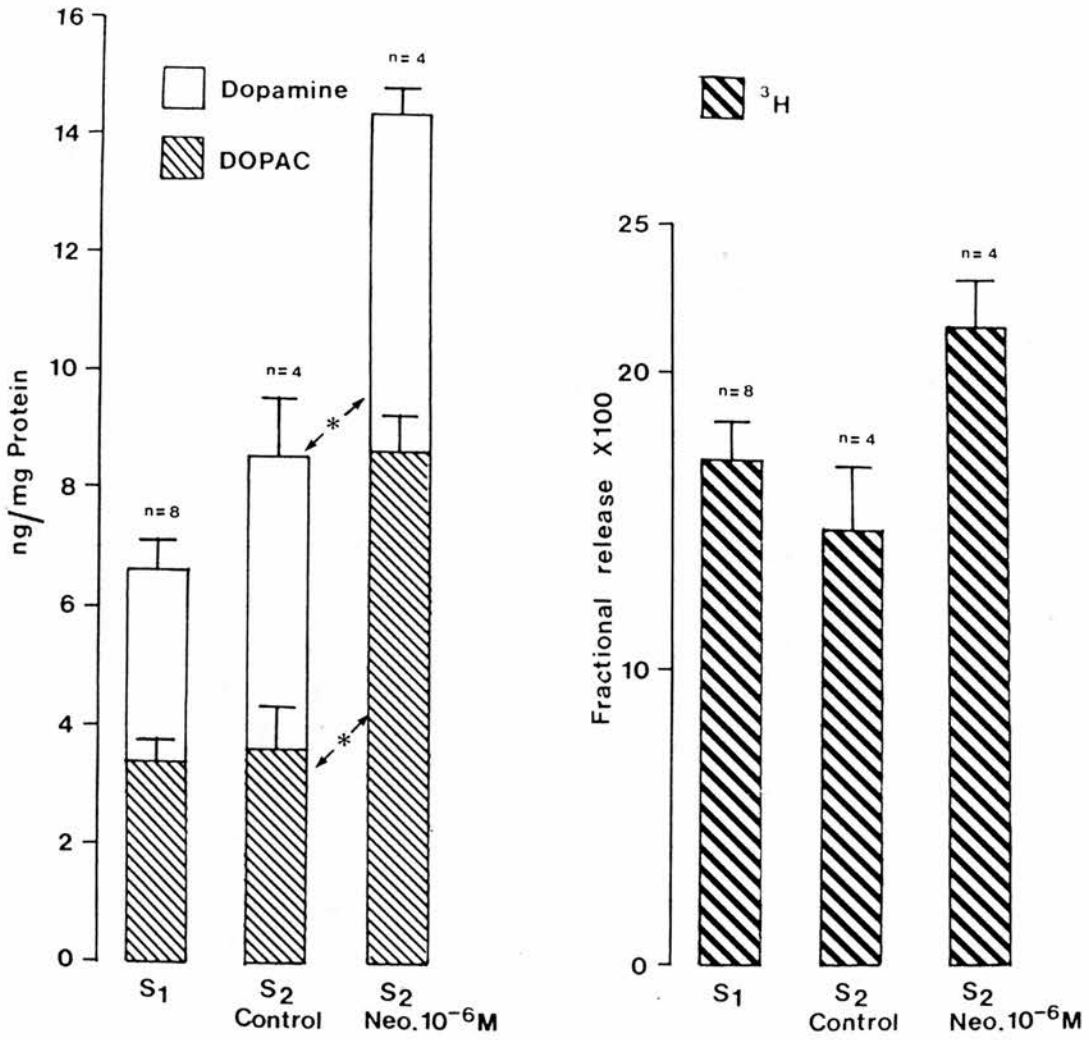


FIGURE 3.14: Comparison of the evoked overflow of endogenous DA and DOPAC with the simultaneous overflow of ^3H.

The 25mM K^+ evoked overflow of endogenous DA and DOPAC at both S_1 and S_2 did not differ from experiments carried out in the absence of ^3H-DA preincubation (see Figure 3.5). Neostigmine (1μM) added to the Krebs solution 30 min after the onset of superfusion enhanced the overflow of both DA and DOPAC to the same extent as in the absence of ^3H-DA preincubation (see Figure 3.9 - S_2/S_1 ratio here being 2.16 ± 0.17 for DA and 2.39 ± 0.16 for DOPAC).

The fractional overflow of ^3H at S_2 was lower than that at S_1 (S_2/S_1 ratio = 0.87) and in the presence of neostigmine the overflow of ^3H increased slightly but not significantly to give an S_2/S_1 ratio of 1.27.

Hence, while there was a more than twofold increase in the evoked overflow of endogenous DA, the simultaneous measurement of ^3H overflow only showed a marginal increase (about 30-40%).

(* $p < 0.05$, Student 't' test, two-tailed)

3.3 Overflow of DA induced by electrical stimulation

(a) Electrical stimulation. Control.

The effect of electrical stimulation (biphasic, square wave pulses of 8-10mA amplitude 2mSec duration, at 20Hz for 30 sec) on striatal slices superfused with Krebs solution is shown in Figure 3.9. The results of evoked overflow are, as with K^+ stimulation, expressed as the increase above basal overflow during the 6 min (3 sample times) after the onset of stimulation. The basal overflow of both DA and DOPAC was the same as that described for K^+ stimulation (see Figure 3.9 and 3.15). Two stimulations, at 16 min (S_1) and 56 min (S_2) from the start of superfusion were applied. The increase in DA overflow at S_1, was found to be 0.44 ± 0.08 (range 0.8 to 0.21) ng/mg protein (means ± s.e.m., n=8), the increase in DOPAC overflow was 2.75 ± 0.20 (range 3.68 to 1.58) ng/mg protein (see Figure 3.16). Hence while there was a much smaller increase in the evoked DA overflow, DOPAC overflow was similar to that observed during 25mM K^+ stimulation. (c.f. Figures 3.5 and 3.16).

At S_2 there was significantly larger overflow of DA, 0.74 ± 0.08 (range 0.92 to 0.24), ng/mg protein giving an S_2/S_1 ratio of 1.80 ± 0.20. The evoked overflow of DOPAC as S_2, 2.93 ± 0.18 (range 3.42 to 1.78) ng/mg protein, did not differ significantly from that of S_1. The ratio of S_2/S_1 for evoked DOPAC was 1.08 ± 0.04 during electrical stimulation, compared to 1.74 ± 0.12 observed during K^+ stimulation (see Figure 3.16).

The DA and DOPAC content of a sample of striatal slices, taken at the end of the preincubation period (before the start of superfusion), was found to be 94.2 ± 3.8 ng/mg protein and 4.8 ± 0.4 ng/mg protein respectively. At the end of the superfusion experiment, these values

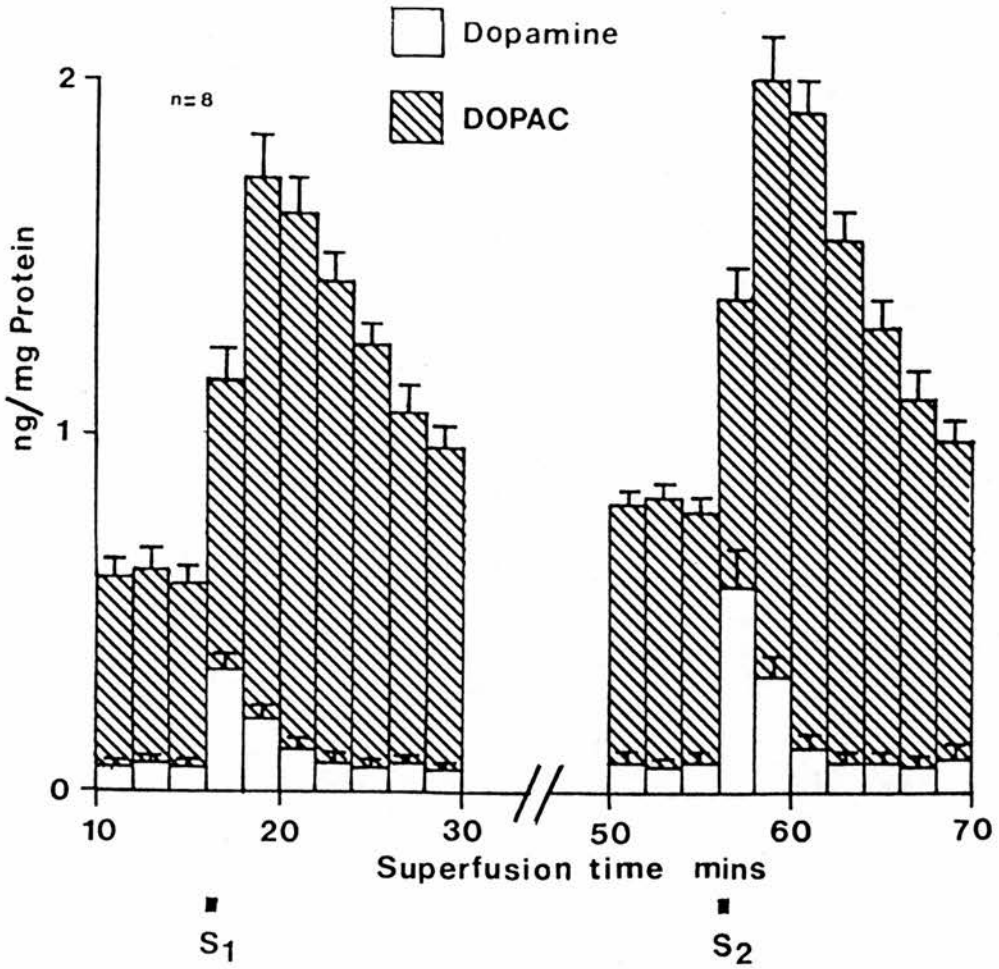


FIGURE 3.15: Striatal slices superfused with Krebs solution were stimulated twice, at S_1 and S_2, electrically. The stimulation parameters were (as with all the other experiments, unless otherwise stated):

2msec pulse duration, 8-10mA amplitude, 20Hz for 30 sec.

The superfusate was collected as 2 min fractions into tubes containing an internal standard and a protective solution. 20-50µl of the superfusate was used for an HPLC-ECD determination of DA and DOPAC. The results are shown above (and summarized overleaf; vertical bars = s.e.m.).

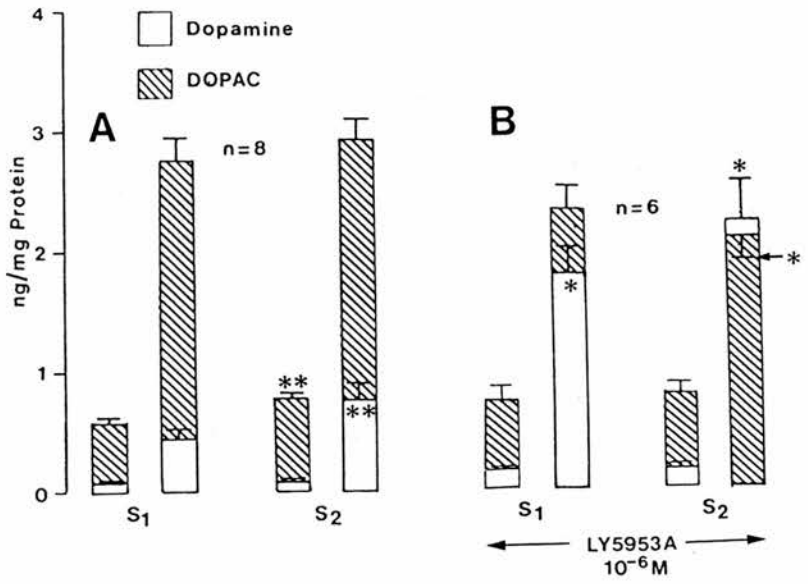


FIGURE 3.16: A. Summary of the control electrically evoked overflow of DA and DOPAC (from Figure 3.15). Each pair of histograms at S_1 and S_2 shows the basal and evoked overflow of DA and DOPAC, for this and all subsequent figures (calculated as in Figure 3.5).

The basal overflow of DOPAC and the evoked overflow of DA at S_2 were significantly higher than at S_1 (**p <0.05, two-tailed, paired Student 't' test).

B. Summary of basal and electrically evoked overflow of DA and DOPAC from striatal tissue superfused with Krebs solution containing 1μM LY5953A from the start of superfusion. While the basal overflow of DA and DOPAC at S_1 and S_2 was not different from control, the evoked overflow of DA was markedly enhanced. DOPAC evoked overflow at S_1 did not differ from control, but at S_2 it was significantly lower (*p <0.05, two-tailed student 't' test).

Ratio of S_2/S_1

Control	DA:	1.80 ± 0.20	DOPAC:	1.08 ± 0.04
LY5953A (1μM)	DA:	1.23 ± 0.06	DOPAC:	0.91 ± 0.08†

(† p <0.05, Wilcoxon Rank test)

DA/DOPAC content of striatal tissue (ng/mg protein, mean ± s.e.m.)

From tissue taken before the start of superfusion.

Start	DA:	94.2 ± 3.8	DOPAC:	4.8 ± 0.4

From tissue taken at the end of experiments.

Control	DA:	176.3 ± 6.9*	DOPAC:	8.6 ± 0.6*
LY5953A (1μM)	DA:	190.7 ± 11.6*	DOPAC:	7.1 ± 1.6*

(* p <0.05, two-tailed Student 't' test)

were about twofold higher, i.e. DA was found to be 176.3 ± 6.9 ng/mg protein and DOPAC was raised to 8.6 ± 0.6 ng/mg protein (n=8).

(b) Tetrodotoxin and Ca^{++} dependence

The evoked overflow of both DA and DOPAC at S_2 was reduced by more than 75% to 0.15 ± 0.05 and 0.22 ± 0.06 (n=4) ng/mg protein respectively in the presence of tetrodotoxin (TTX, added to Krebs solution 26 min before S_2). Addition of TTX (1μM) did not have any significant effect on the basal overflow of either DA or DOPAC. Similar effects were seen when the tissues were superfused with Ca^{++} free (+0.1mM EGTA) Krebs at S_2 (see Figure 3.17).

(c) The effect of uptake inhibition on the electrically evoked overflow of DA and DOPAC

Addition of the uptake inhibitor LY5953A (1μM) increased the basal overflow of DA by twofold from that seen in the absence of drugs to 0.16 ± 0.02 ng/mg protein/2 min, there was, however, no significant change in the basal overflow of DOPAC. The evoked overflow of DA at S_1 was increased by about four fold to 1.81 ± 0.22 (range 2.66 to 0.95) ng/mg protein (n=6), without any significant change in the evoked overflow of DOPAC (see Figure 3.16). During S_2, the evoked overflow of DA was 2.32 ± 0.35 (range 3.96 to 1.2) ng/mg protein, giving an S_2/S_1 ratio of 1.23 ± 0.06. At S_2, the evoked overflow of DOPAC, 2.09 ± 0.23 (range 2.95 to 1.04) ng/mg protein, was significantly lower than the control values (see Figure 3.16) giving an S_2/S_1 ratio of 0.91 ± 0.08.

The tissue content of DA at the end of experiments conducted in the presence of 1μM LY5953A, was found to be 190.7 ± 11.6 ng/mg protein, the DOPAC content was 7.1 ± 1.6 ng/mg protein, and neither differed significantly from control values.

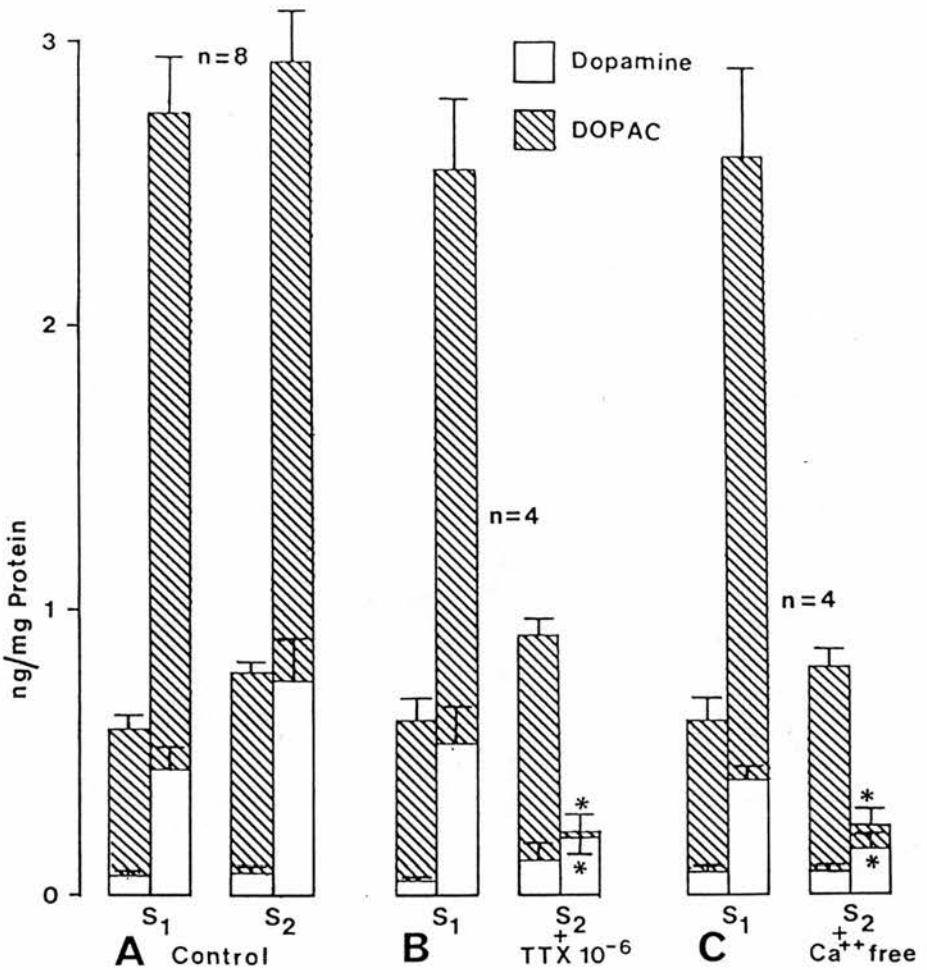


FIGURE 3.17: A. The control basal and electrically evoked overflow of DA and DOPAC (as in Figure 3.16).

B. Tetrodotoxin (1μM) was added to the Krebs solution superfusing striatal slices, 30 min after the onset of superfusion, i.e. after S_1. The presence of TTX did not affect the basal overflow of either DA or DOPAC before S_2, however, the evoked overflow of DA and DOPAC was reduced by more than 75%.

C. 30 min after the onset of superfusion and after the control S_1, the striatal slices were superfused with Ca^{++} free (+0.1mM EGTA) Krebs. The basal overflow of DA and DOPAC before S_2 remained unaffected but the evoked overflow of DA and DOPAC at S_2 was reduced by more than 75%.

(* $p < 0.01$, two-tailed, Student 't' test)

(d) The effect of a unilateral 6-OHDA lesion

Seven to 14 days after a unilateral 6-OHDA lesion of the striato-nigral pathway, the rats were tested for a successful lesion by behavioural analysis. Only rats which turned contralateral to the lesion site (200 or more turns in 30 min) when challenged with 0.3 mg/kg apomorphine (I.P.), were used. Two to 4 days after the behavioural analysis, rats were sacrificed and the striata used for superfusion experiments to study the overflow of endogenous DA/DOPAC on electrical stimulation (8-10mA, 20Hz, 30 sec). The uptake inhibitor, LY5953A (1μM), was added to the superfusing solution 26 min before the onset of S_2. The lesioned striatum and the contralateral control striatum from each animal were superfused in separate chambers. While the basal and evoked overflow of both DA and DOPAC from the contralateral striatum did not differ from control values (see Figure 3.18 and Figure 3.19 cf. 3.16), there was no detectable or evoked overflow of either DA or DOPAC from the lesioned striatum, at S_1 or S_2 despite the presence of LY5953A (1μM).

(e) Frequency dependence

Striatal slices superfused with Krebs solution (as before) were stimulated twice at 8-10mA, for 200 pulses at 2, 10, 20 or 50Hz in a random sequence. These experiments were carried out in the presence of nomifensine (1μM) present from the start of superfusion. The results (calculated as above) averaged at each frequency are tabulated on page 96 (mean ± s.e.m., see Figure 3.20).

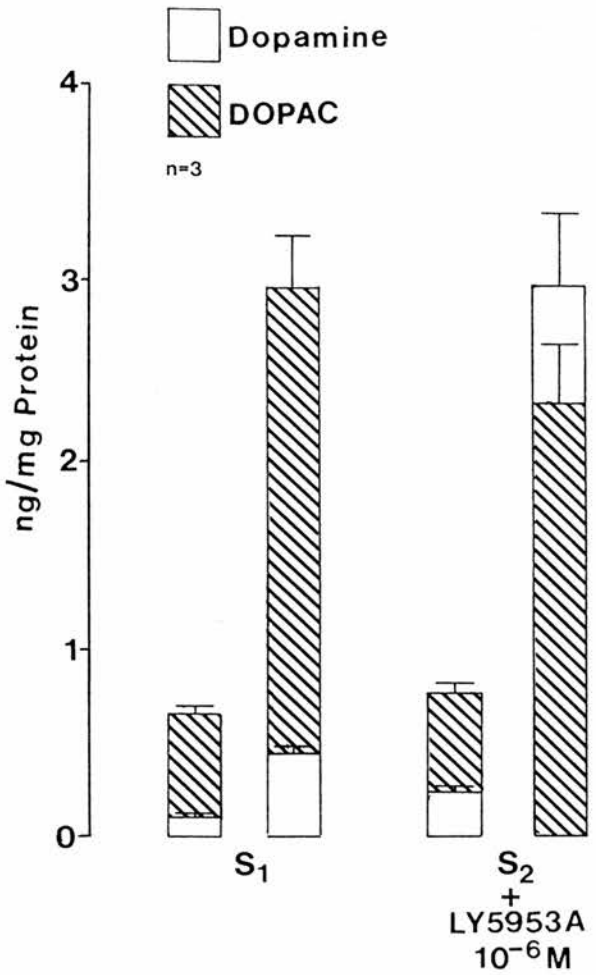


FIGURE 3.18: The basal and evoked overflow of DA and DOPAC from the unlesioned side of a unilaterally 6-OHDA lesioned rat.

At S_1, electrical stimulation evoked the overflow of DA and DOPAC from the unlesioned striatum superfused in Krebs solution, values were not different from control experiments (see Figure 3.16A).

30 min after the onset of superfusion, 1μM LY5953A was added to the Krebs solution. The basal and evoked overflow of DA/DOPAC at S_2 was similar to that found during control experiments (see Figure 3.16B).

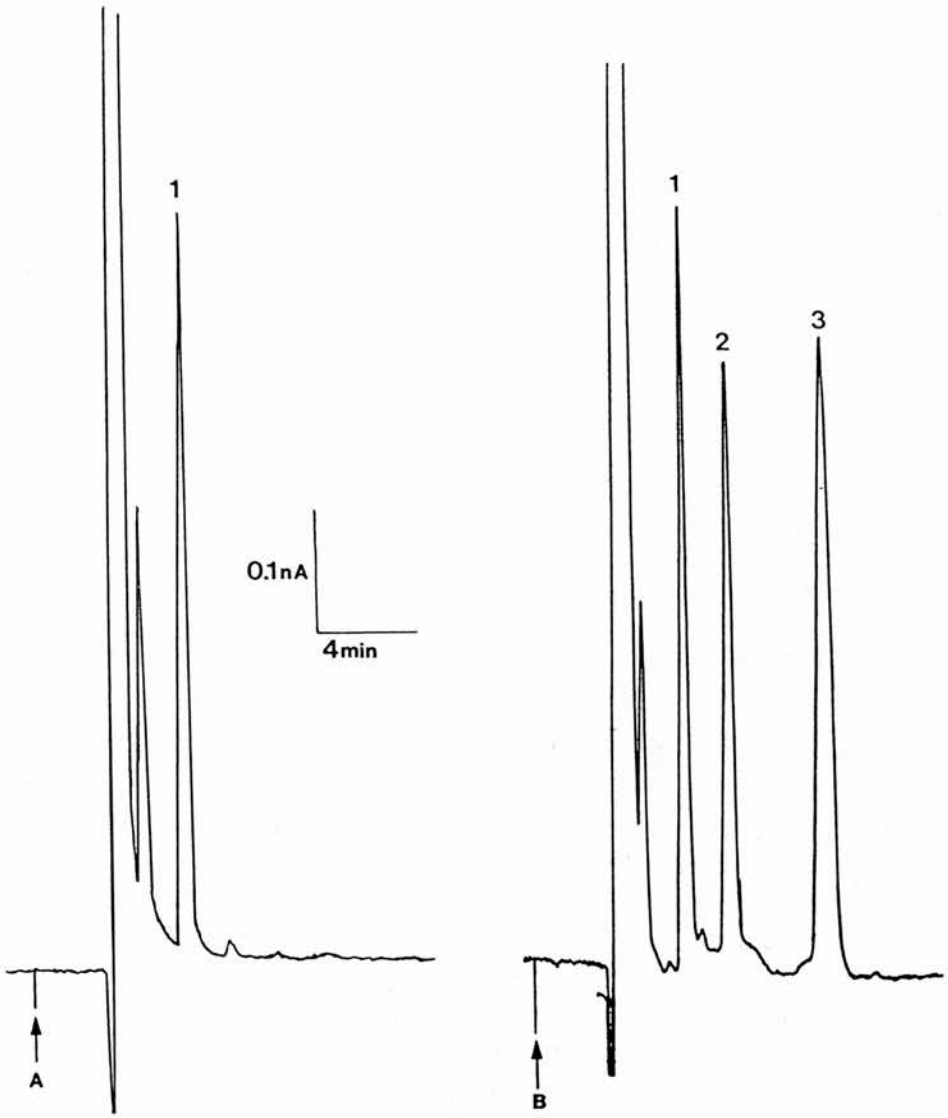


FIGURE 3.19: Comparison of the HPLC-ECD traces of superfusate samples (collected during S_2, i.e. at 56 min) from A: the lesioned, and B: the unlesioned striatal slices of a unilaterally 6-OHDA lesioned rat. The DA/DOPAC content of superfusate samples collected during the second electrical stimulation, S_2, in the presence of 1μM LY5953A, are shown above.

1 = Dihydroxybenzylamine, internal standard.
2 = Dopamine.
3 = Dihydroxybenzylamine (DOPAC).

No detectable DA or DOPAC was found in the lesioned side, even during electrical stimulation (A), while the overflow of DA and DOPAC from the unlesioned side (B) did not differ from control (see text).

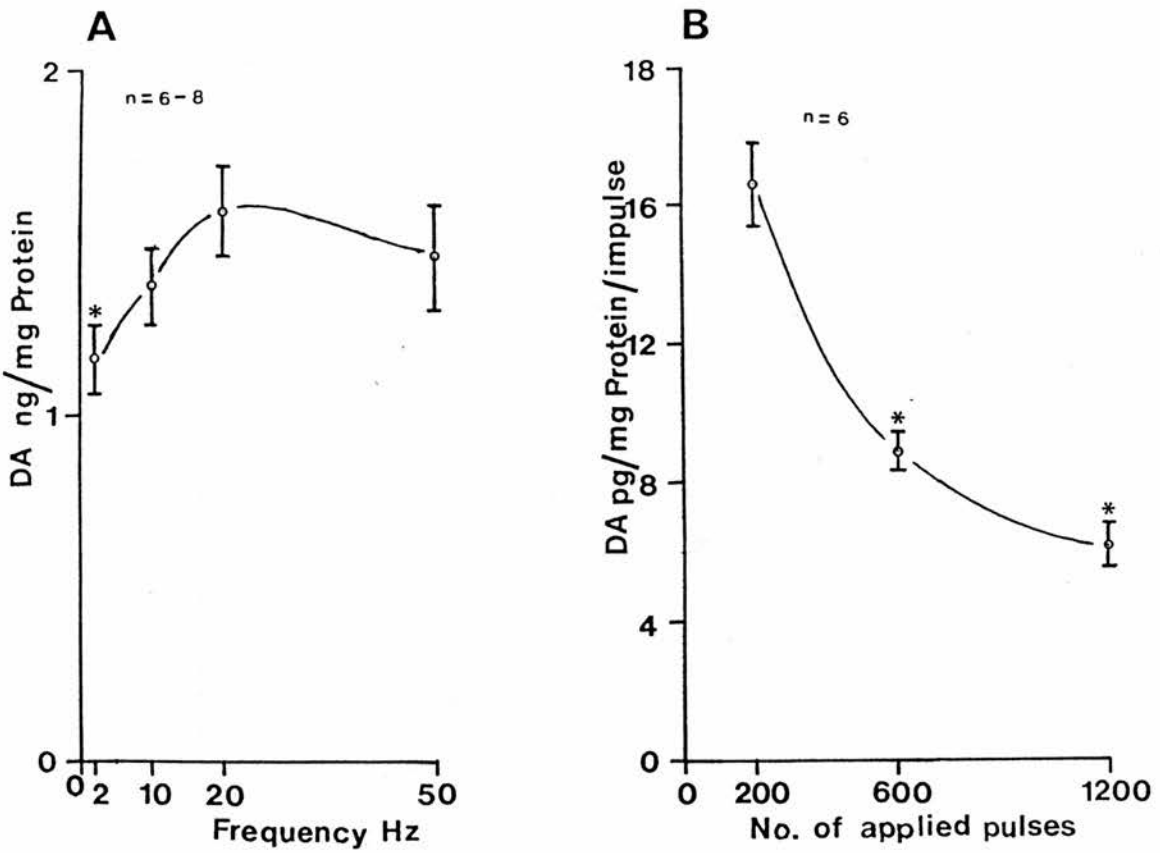


FIGURE 3.20: A. Striatal slices superfused with Krebs solution containing 1μM, nomifensine, were stimulated twice (S_1 and S_2) at 2, 10, 20 or 50Hz for 200 pulses (all other parameters remaining unaltered), in a random sequence. The results for the evoked overflow of DA were calculated as in Figure 3.5, and plotted against the stimulating frequency (see table of results on page 96). The evoked overflow of DA, during the 200 applied pulse, appeared to increase with increasing frequency of stimulation up to 20Hz.

B. Striatal slices superfused with Krebs solution containing 5μM LY5953A, were stimulated once for 10, 30 or 60 sec at 20Hz (i.e. for 200, 600 or 1200 pulses at 20Hz). The results of the evoked overflow of DA calculated as in Figure 3.5 were divided by the number of pulses applied and plotted against the applied number of pulses. The results show that DA overflow per impulse decreases with increasing number of pulses applied.

(* $p < 0.05$, two-tailed Student 't' test, indicates values significantly different from those at 20Hz, 200 pulse stimulation.)

Frequency (Hz)	DA (ng/mg protein	DOPAC (ng/mg protein	n
2	1.16 ± 0.10*	1.82 ± 0.17	8
10	1.37 ± 0.11	1.89 ± 0.19	6
20	1.59 ± 0.13	1.94 ± 0.16	8
50	1.45 ± 0.15	1.95 ± 0.20	6

*Significantly different from values obtained at 20Hz stimulation ($p < 0.05$ student "t" test, two-tailed).

Keeping the number of pulses constant (i.e. 200), the evoked release of DA and DOPAC increased from that seen at 2Hz to a maximum during 20Hz stimulation. Preliminary experiments at 100 or 200Hz stimulation indicated that the release was not increased with increasing frequency after 20Hz.

(f) DA overflow per impulse

In order to see the effect on the evoked overflow of DA per impulse, at a fixed frequency of 20Hz, striatal slices were stimulated once for 10, 30 or 60 sec. The uptake inhibitor LY5953A (5μM) was added to the Krebs solution from the start of superfusion. The results calculated as before, were divided by the number of applied pulses and expressed as overflow of DA ng/mg protein/impulse. The results (Figure 3.20) show that the increase in the number of pulses applied decreases the DA overflow per impulse.

(g) The effect of monoamine oxidase inhibition

Pargyline (1μM) added to the Krebs solution, 26 min before S_2, reduced the basal overflow of DOPAC before S_2 to 0.19 ± 0.02 (range 0.24 to 0.13) ng/mg protein/2 min (n=4, see Figure 3.21). The overflow of DA in the presence of pargyline was not significantly

different from control, i.e. 0.63 ± 0.08 (range 0.8 to 0.4) ng/mg protein, however, the evoked overflow of DOPAC was completely inhibited. At the end of the experiments, the tissue content of DA was found to be significantly higher, i.e. 201.3 ± 4.0 ng/mg protein.

(h) The effect of 'in vivo' tyrosine hydroxylase inhibition

Rats pretreated with α-methyl-para-tyrosine (AMPT), 250mg/kg (I.P.), for 2 hr were sacrificed and the striata removed and superfused as before. The uptake inhibitor, LY5953A (1μM), was added to the Krebs solution from the start of superfusion. No detectable basal or evoked overflow of either DA or DOPAC was found. At the end of the experiments there was no detectable DOPAC in the tissues; DA content was found to be 40.8 ± 5.2 ng/mg protein (n=3).

(i) The effect of 'in vitro' tyrosine hydroxylase inhibition

AMPT (0.5mM) added to the Krebs solution from the start of superfusion of striatal slices. Under these conditions, DA basal overflow was found to be below detectable levels in most experiments, and the basal DOPAC overflow was reduced by about half from control values to 0.29 ± 0.05 (range 0.41 to 0.15) ng/mg protein (n=4). A similar reduction was also found in the evoked overflow of DA (0.21 ± 0.02, range 0.29 to 0.19 ng/mg protein) at S_1. The evoked overflow of DOPAC at S_1 was found to be 0.19 ± 0.03 (range 0.2 to 0.08) ng/mg protein. Compared to control values, the basal and evoked overflow of both DA and DOPAC were also reduced (see Figure 3.21), by more than 70%.

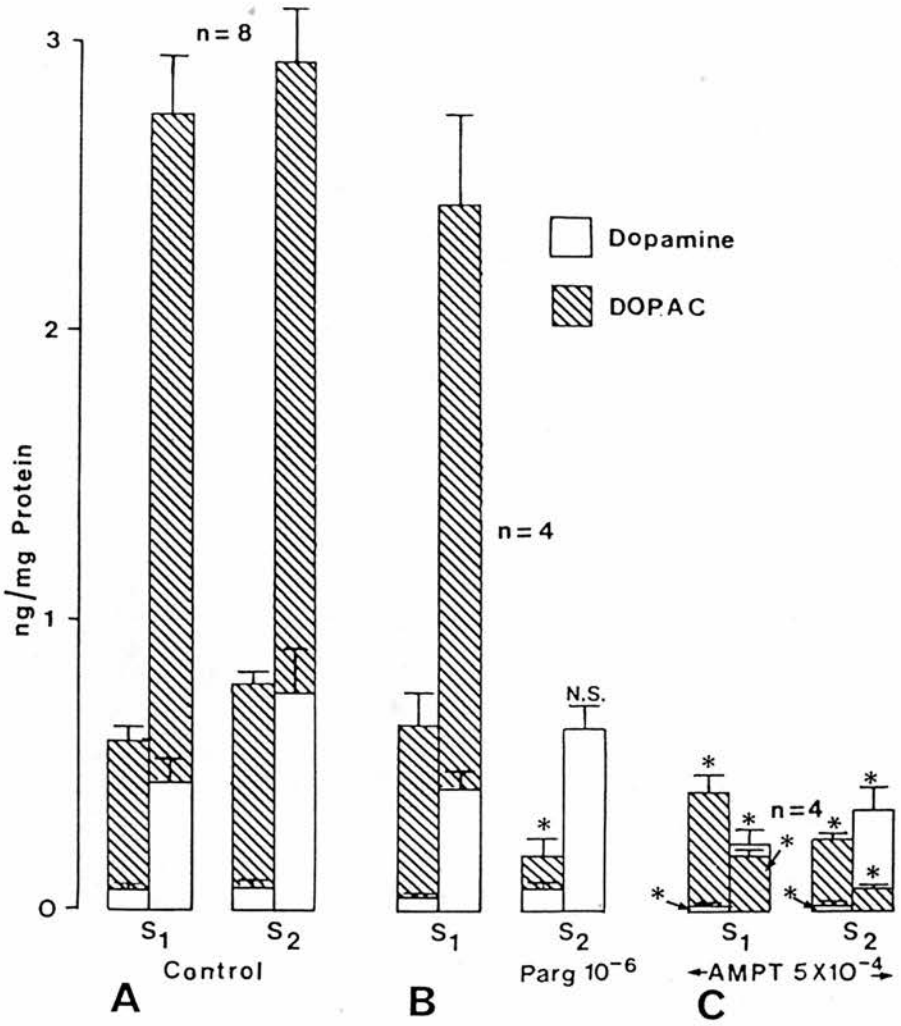


FIGURE 3.21: A. The basal and electrically evoked overflow of DA and DOPAC under control conditions, from Figure 3.16.

B. Pargyline (1μM) added to the Krebs solution 30 min after onset of superfusion significantly reduced the basal overflow of DOPAC before, and abolished the evoked overflow of DOPAC, at S_2. The basal and evoked overflow of DA during S_2, however, were unaltered.

C. AMPT (0.5mM) added to the Krebs solution from the start of superfusion, decreased the basal overflow of DA and DOPAC before S_1. The evoked overflow of DA was also reduced by about 50%, and the evoked DOPAC overflow at S_1 was only 6% of control levels.

The basal overflow before S_2 was reduced further. At S_2 the evoked overflow of DA was reduced by 70% and that of DOPAC was almost abolished.

Tissue content of DA and DOPAC at the end of experiments (ng/mg protein)

Control	DA:	176.3 ± 6.9	DOPAC:	8.6 ± 0.6
Pargyline	DA:	201.3 ± 4.0*	DOPAC:	0.23± 0.09*
AMPT	DA:	90.8 ± 6.7*	DOPAC:	1.9 ± 0.5*

(* $p < 0.05$, two-tailed Student 't' test, indicating values significantly different from respective control values.)

(j) The influence of muscarinic agents on the electrically evoked overflow of DA and DOPAC

i) Indirectly acting muscarinic agonist:

Striatal slices superfused with Krebs solution were stimulated twice (S_1 and S_2) at 20Hz for 30 sec, as before. Neostigmine (1μM) and tubocurarine (1μM) added 30 min after the onset of superfusion, increased the evoked overflow of DA to 1.95 ± 0.24 (range 3.17 to 1.02) ng/mg protein (n=10). The evoked overflow of DOPAC was also increased compared to the internal control (S_1) from 2.10 ± 0.17 (range 3.01 to 1.45) ng/mg protein at S to 2.72 ± 0.21 (range 3.82 to 1.72) ng/mg protein (paired student "t" test, $p < 0.05$). The S_2/S_1 ratio for DA overflow was found to be 3.71 ± 0.47 and 1.31 ± 0.05 for DOPAC; these values were significantly different from control by non-parametric analysis (see Figure 3.22). The basal overflow of DA and DOPAC remained unaffected by the drugs, but at the end of the experiment, the tissue content of DA was about 30% less than the control value, i.e. 133.6 ± 6.6 ng/mg protein, with a smaller reduction in the DOPAC content to 6.8 ± 0.87 ng/mg protein (not significantly different from control).

Addition of atropine (1μM) with neostigmine (1μM) before S_2 resulted in a significantly smaller evoked overflow of DA, 0.30 ± 0.03 (range 0.4 to 1.8) ng/mg protein (n=7) and DOPAC, 1.36 ± 0.32 (range 3.07 to 0.8) ng/mg protein. While the basal overflow of DA and DOPAC, and the DA tissue content at the end of the experiment were not significantly different from control (see Figure 3.22), the DOPAC content was lower, i.e. 4.56 ± 0.36 (range 5.2 to 3.0) ng/mg protein.

FIGURE 3.22: A. The basal and electrically evoked overflow of DA and DOPAC under control conditions, from Figure 3.16.

B. After the internal control stimulation, S_1, neostigmine (1μM) and tubocurarine (1μM) was added to the superfusing Krebs solution (at 30 min). The basal overflow of DA and DOPAC before S_2 remained unaltered. The evoked overflow of DA and DOPAC was markedly enhanced at S_2.

C. After the internal control stimulation, S_1, neostigmine (1μM) and atropine (1μM) was added to the superfusing Krebs solution (at 30 min). While the basal overflow of DA and DOPAC remained unaltered, a reduction in the evoked overflow of DA and DOPAC was found.

S_2/S_1 ratios for the evoked overflow of DA and DOPAC

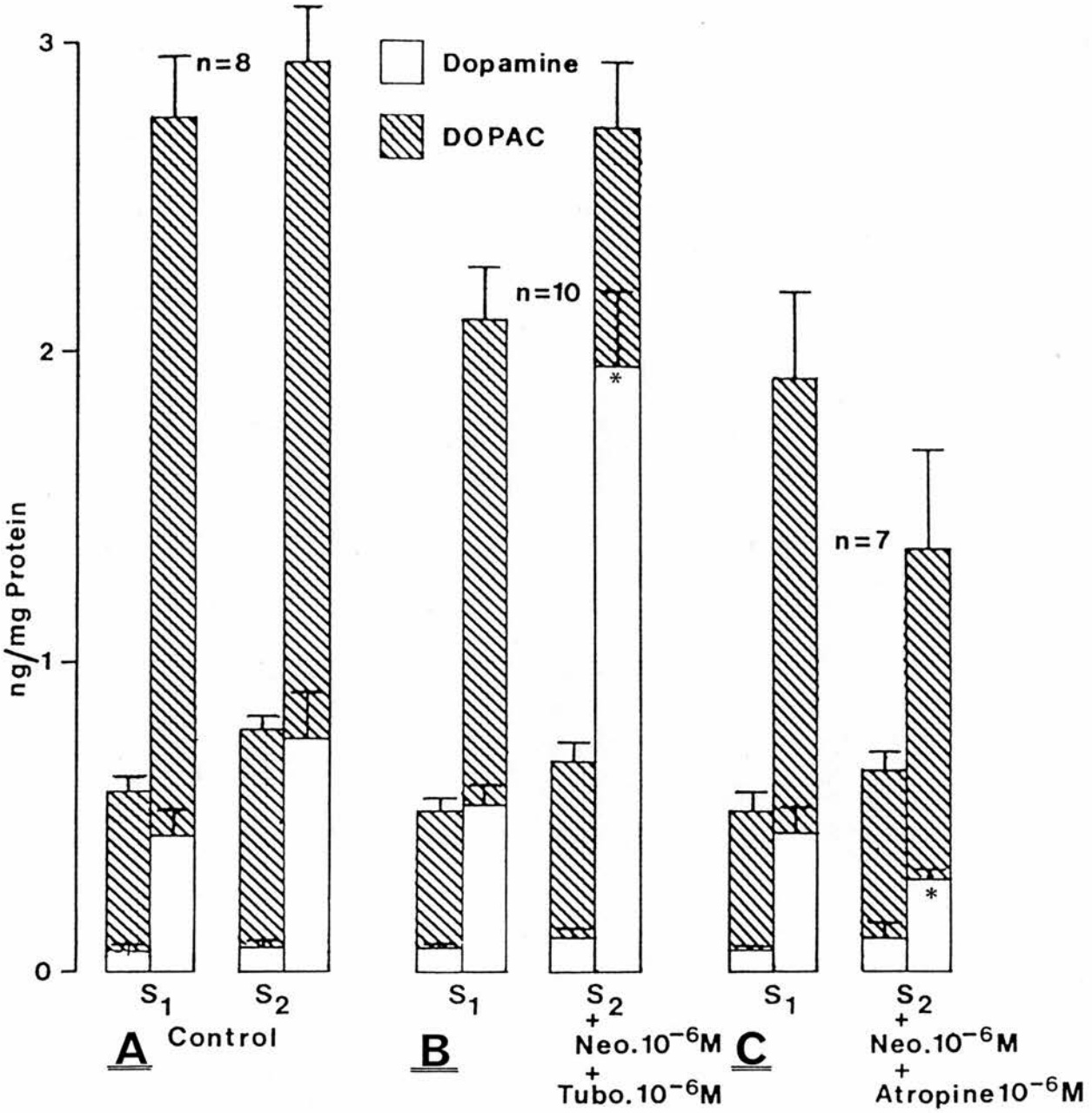
Control	DA: 1.80 ± 0.20	DOPAC: 1.08 ± 0.04
Neostigmine + Tubocurarine	DA: 3.71 ± 0.47†	DOPAC: 1.31 ± 0.05†
Neostigmine + Atropine	DA: 0.75 ± 0.12†	DOPAC: 0.70 ± 0.09†

(† $p < 0.05$, Wilcoxin Rank test)

DA/DOPAC content of tissues at the end of the experiments (ng/mg protein)

Control	DA: 176.3 ± 6.9	DOPAC: 8.6 ± 0.6
Neostigmine + Tubocurarine	DA: 133.6 ± 6.6*	DOPAC: 6.8 ± 0.9
Neostigmine + Atropine	DA: 155.8 ± 8.4	DOPAC: 4.5 ± 0.6*

(* $p < 0.05$ two-tailed, Student 't' test)


3
2
1
0
ng/mg Protein
n=8
n=10
n=7
Dopamine
DOPAC
*
*
S1
S2
S1
S2
S1
S2
A Control
B
Neo.10⁻⁶M + Tubo.10⁻⁶M
C
Neo.10⁻⁶M + Atropine10⁻⁶M

ii) Directly acting muscarinic agonists:

Oxotremorine (5μM) added to the Krebs solution 30 min after onset of superfusion caused an almost threefold increase in the evoked overflow of DA at S_2, i.e. 2.17 ± 0.27 (range 3.11 to 1.59) ng/mg protein (n=6). DOPAC overflow at S_2, at 2.76 ± 0.25 (range 3.77 to 2.0) ng/mg protein, was 30% higher than that observed at S_1 (2.12 ± 0.19, range 3.11 to 1.7, ng/mg protein), giving an S_2/S_1 ratio of 1.31 ± 0.06 (significantly different from control, see Figure 3.23). The basal overflow of both DA and DOPAC remained at control levels. As with the indirectly acting muscarinic agents (above), oxotremorine significantly reduced the DA content (145.8 ± 13.4 ng/mg protein) of the tissue at the end of the experiment, while DOPAC (8.4 ± 1.4 ng/mg protein) remained unaltered.

iii) The effect of neostigmine on the overflow of DA and DOPAC in the presence of the uptake inhibitor, LY5953A:

In order to determine whether the effect of the acetylcholine esterase inhibitor was due to an increase in the overflow or in the release of DA, neostigmine (1μM) was added to the Krebs solution 30 min after onset of superfusion in the presence of an uptake inhibitor. As described before, LY5953A (1μM) was present from the start of superfusion. The presence of neostigmine, with the uptake inhibitor LY5953A further enhanced the overflow of DA at S_2 (see Figure 3.24) to 3.15 ± 0.38 (range 4.13 to 1.74) ng/mg protein (n=6), the evoked rise in DOPAC overflow was similarly increased to 2.74 ± 0.18 (range 3.73 to 2.44) ng/mg protein at S_2. The basal overflow did not differ from that in the presence of LY5953A alone. As was found in the absence of uptake inhibitors (see Figure 3.22), the tissue content of DA at the end of the experiment was significantly lower in the additional

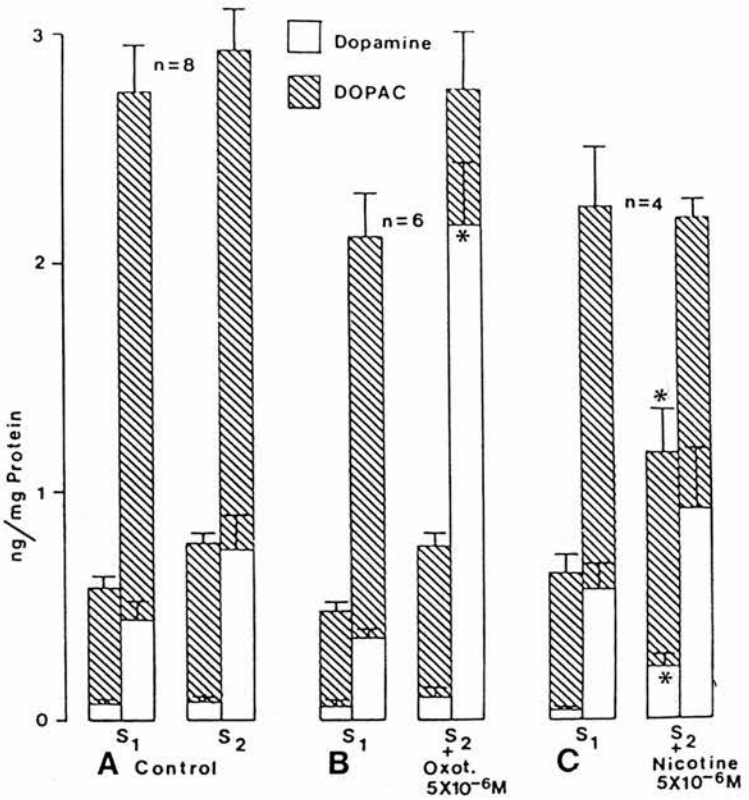


FIGURE 3.23: A. The basal and evoked overflow of DA and DOPAC under control conditions, from Figure 3.16.

B. Oxotremorine (Oxot.) (1μM) enhanced the evoked overflow of DA and DOPAC at S_2 (added again 30 min after start of superfusion). The basal overflow remained unaltered.

C. Nicotine (1μM) enhanced only the basal overflow of DA and DOPAC before S_2 (added at 30 min), while the evoked overflows remained unchanged.

S_2/S_1 ratios of the evoked overflow of DA and DOPAC

Control	DA:	1.80 ± 0.20	DOPAC:	1.08 ± 0.04
Oxot.	DA:	6.27 ± 0.74†	DOPAC:	1.31 ± 0.05†
Nicotine	DA:	1.51 ± 0.15	DOPAC:	1.02 ± 0.09

DA/DOPAC content of the tissues at the end of the experiments

(ng/mg protein)

Control	DA:	176.3 ± 6.9	DOPAC:	8.6 ± 0.6
Oxot.	DA:	145.8 ± 13.4*	DOPAC:	8.4 ± 1.4*
Nicotine	DA:	161.0 ± 13.6	DOPAC:	9.4 ± 0.2

(* $p < 0.05$, two-tailed Student 't' test)

(† $p < 0.05$, Wilcoxin Rank test)

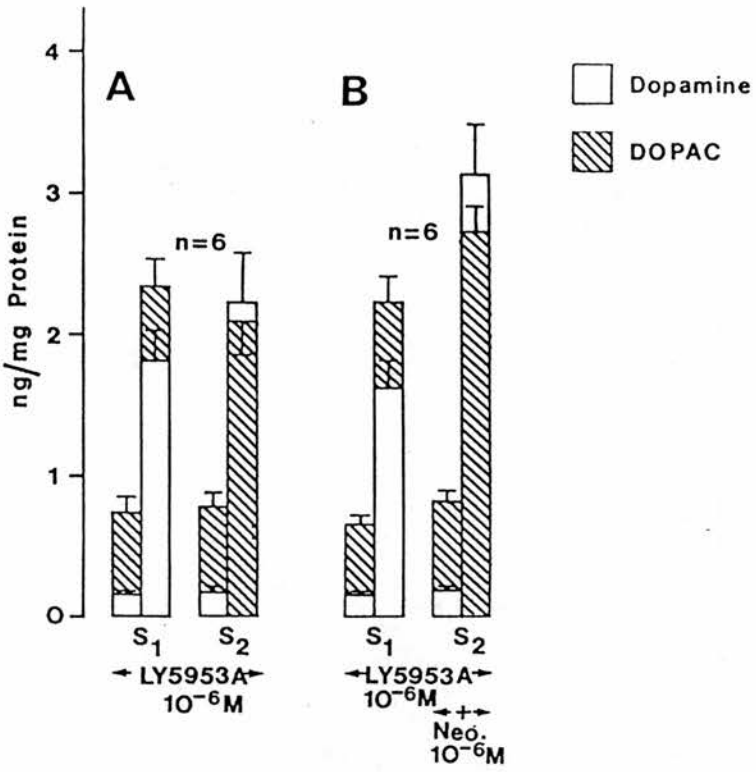


FIGURE 3.24: A. The basal and evoked overflow of DA and DOPAC in the presence of LY5953A (1μM), from Figure 3.16.

B. Neostigmine (1μM) added 30 min after the onset of superfusion in the presence of 1μM LY5953A, further enhanced the evoked overflow of DA and increased the evoked overflow of DOPAC. The basal overflow remained unaltered.

S_2/S_1 ratios for the evoked overflow of DA and DOPAC

LY5953A	DA: 1.23 ± 0.06	DOPAC: 0.91 ± 0.08
LY5953A + neostigmine	DA: 1.95 ± 0.13†	DOPAC: 1.24 ± 0.07†

DA/DOPAC content of the tissues at the end of the experiments (ng/mg protein)

LY5953A	DA: 190.7 ± 11.6	DOPAC: 7.1 ± 1.6
LY5953A + neostigmine	DA: 148.4 ± 5.9*	DOPAC: 6.2 ± 0.8

(* $p < 0.05$, two-tailed Student 't' test)

(† $p < 0.05$, Wilcoxin Rank test)

presence of neostigmine at S_2 compared to LY5953A only (see Figure 3.24) while DOPAC remained unaltered.

(k) The effect of a nicotinic agonist

Nicotine (5μM) added to the superfusing Krebs solution 30 min after the onset of superfusion did not significantly alter the evoked overflow of either DA or DOPAC (see Figure 3.23). The basal overflow of DA was increased to 0.23 ± 0.05 ng/mg protein/2 min, as was the basal overflow of DOPAC to 1.17 ± 0.29 ng/mg protein/ 2 min at S_2.

(l) The effect of dopaminergic agents on the evoked overflow of DA and DOPAC

i) Dopaminergic agonist effects:

The dopaminergic agonist, 3PPP [3-(3-hydroxyphenyl)-N-n-propylpiperidin] at a dose of 5μM added to the superfusing Krebs solution 30 min after the onset of superfusion, decreased the evoked overflow at S_2, of both DA, to 0.35 ± 0.02 (range 0.4 to 0.28) ng/mg protein (n=4), and DOPAC to 1.87 ± 0.24 (range 2.67 to 1.40) ng/mg protein. The S_2/S_1 ratios for both evoked DA and DOPAC in the presence of 3PPP were significantly different from control (see Figure 3.25). The basal overflow of DA before S_2 was unaffected by 3PPP, however, the basal overflow of DOPAC at the same point was about 30% higher than control levels (i.e. 1.06 ± 0.14 ng/mg protein/2 min). The S_2/S_1 ratio for basal DOPAC was significantly higher than control.

ii) The effect of the dopaminergic agonist, 3PPP in the presence of an uptake inhibitor:

LY5953A (5μM) present from the start of superfusion greatly enhanced the basal overflow of DA (to 0.23 ± 0.02 ng/mg protein/2 min) and the evoked overflow of DA, both at S_1, i.e. 5.19 ± 0.50

FIGURE 3.25: A. The basal and evoked overflow of DA and DOPAC under control conditions, from Figure 3.16.

B. The D_2 agonist 3PPP (5μM) added at 30 min of superfusion decreased the evoked overflow of DA and DOPAC. The basal overflow of DOPAC was also enhanced in the presence of 3PPP.

C. Haloperidol (0.01μM) enhanced the basal overflow of both DA and DOPAC. The evoked overflow of DA and DOPAC at S_2 were also larger.

S_2/S_1 ratios of the evoked overflow of DA and DOPAC

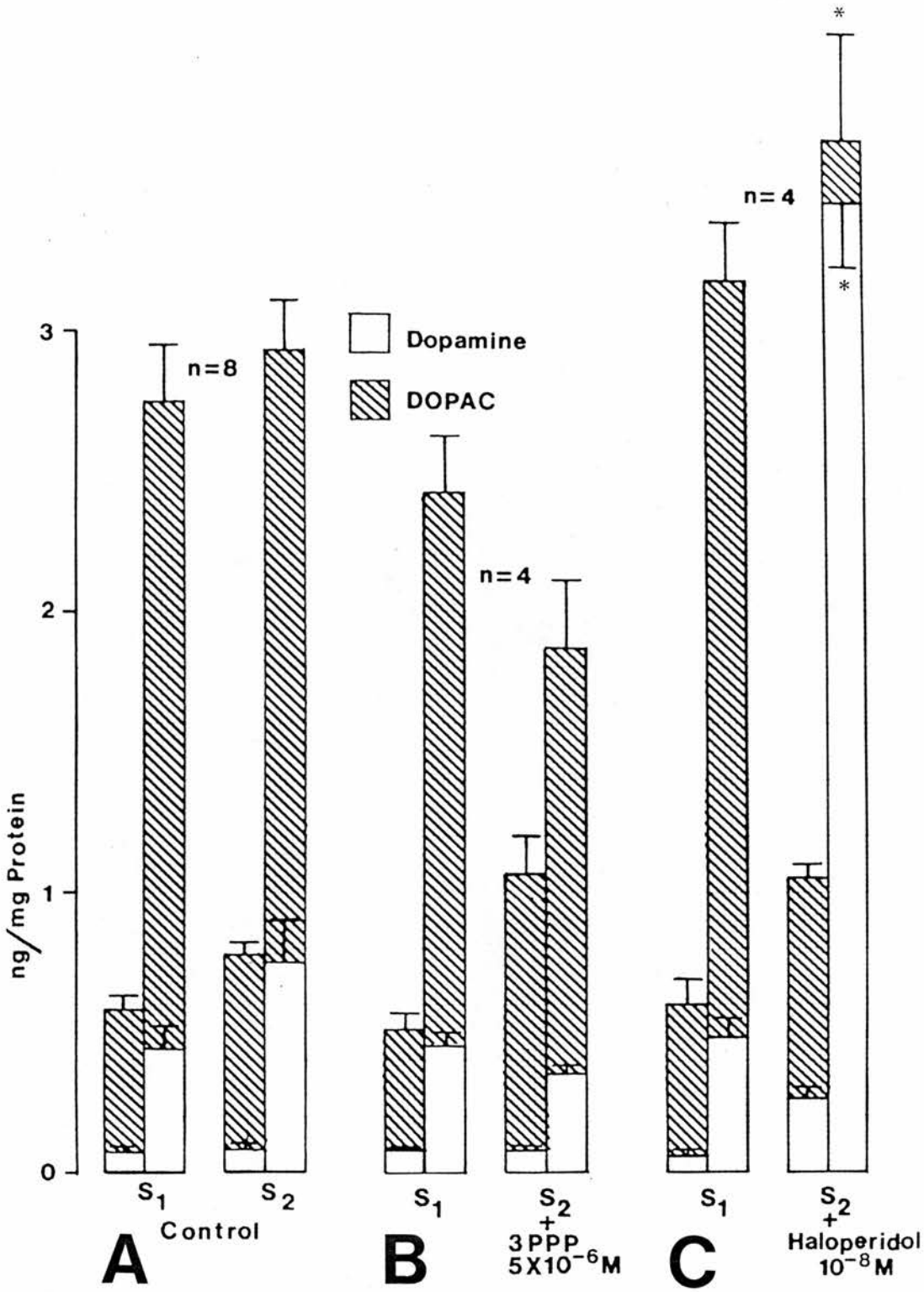
Control	DA:	1.80 ± 0.10	DOPAC:	1.08 ± 0.04
3PPP	DA:	0.80 ± 0.09†	DOPAC:	0.77 ± 0.06†
Haloperidol	DA:	7.57 ± 0.68†	DOPAC:	1.21 ± 0.02†

DA/DOPAC content of the tissues at the end of the experiments (ng/mg protein)

Control	DA:	176.3 ± 6.9	DOPAC:	8.6 ± 0.61
3PPP	DA:	148.8 ± 2.6*	DOPAC	6.23 ± 0.71
Haloperidol	DA:	120.3 ± 6.16*	DOPAC:	11.3 ± 0.88

(* $p < 0.05$, two-tailed Student 't' test)

(† $p < 0.05$, Wilcoxin Rank test)


Dopamine
DOPAC
ng/mg Protein
3
2
1
0
n=8
n=4
n=4
*
*
S1
S2
S1
S2
+
3PPP
5X10-6M
S1
S2
+
Haloperidol
10-8M
A
Control
B
C

(range 6.63 to 4.05) ng/mg protein (n=4) and at S_2, i.e. 5.80 ± 0.23 (range 6.63 to 5.05) ng/mg protein compared to that seen in the presence of 1μM LY5953A (cf. Figure 3.26 and 3.16).

The basal and evoked overflow of DOPAC remained unaltered from those seen at 1μM LY5953A (see Figure 3.16). However, the DA content of the tissues in the presence of 5μM LY5953A was significantly reduced to 138.0 ± 9.8 ng/mg protein.

The addition of 5μM 3PPP at S_2, in the presence of 5μM LY5953A, reduced the evoked overflow of DA to 4.23 ± 0.57 ng/mg protein (n=4) and DOPAC to 1.65 ± 0.14 ng/mg protein. The DA/DOPAC content of the tissues in the presence of 3PPP and LY5953A (both at 5μM) did not differ from that seen in the presence of only 5μM LY5953A (see Figure 3.26).

iii) Dopaminergic antagonist effects:

The addition of haloperidol (0.01μM) before S_2 greatly enhanced both the basal and the evoked overflow of DA at S_2 (see Figure 3.25). The evoked overflow of DA was increased by more than four fold to 3.11 ± 0.5 (range 4.39 to 2.80) ng/mg protein (n=4). Both the basal and evoked overflow of DOPAC were also increased; the 25% above control increase in evoked DOPAC raised this value to 3.69 ± 0.38 (range 4.56 to 2.8) ng/mg protein.

As with 3PPP, haloperidol also decreased the tissue content of DA at the end of the experiment to 120.3 ± 6.2 ng/mg protein, however, in this case DOPAC was raised to 11.3 ± 0.88 ng/mg protein.

Preliminary experiments with higher doses of haloperidol (1μM) showed no increase in either the basal or evoked (0.61 ng/mg protein, n=2) overflow of DA at S_2. The basal overflow of DOPAC was greatly increased (2.7 ng/mg protein/2 min) with a marked increase in the

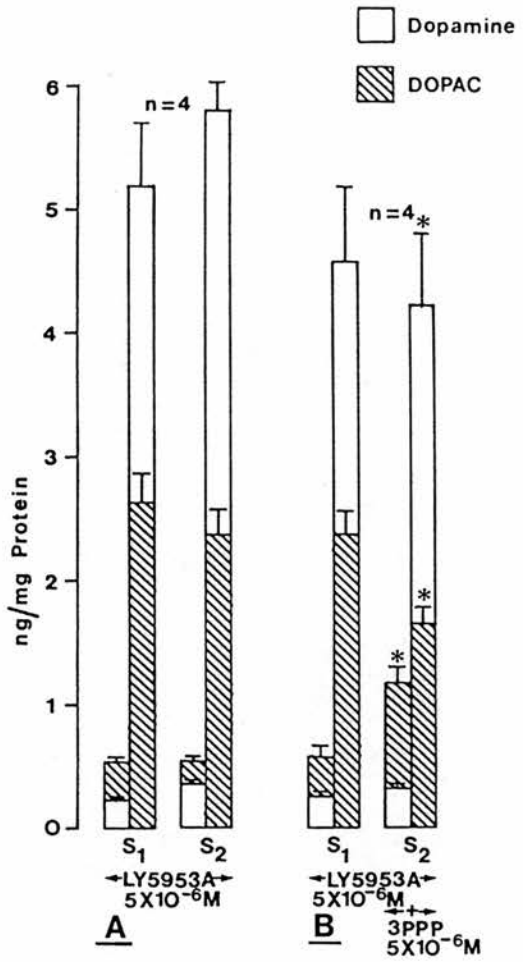


FIGURE 3.26: A. The basal and evoked overflow of DA and DOPAC in the presence of 5μM LY5953A. The basal and evoked overflow of DA, both at S_1 and S_2 are significantly higher ($p < 0.05$, Student 't' test, two-tailed) than in the presence of 1μM LY5953A (see Figure 3.16).

B. 3PPP (5μM) added at 30 min, enhanced the basal overflow of DOPAC, in the presence of the uptake inhibitor LY5953A (5μM).

The evoked overflow of DA and DOPAC at S_2 was significantly reduced.

DA/DOPAC content of the tissues at the end of the experiments (ng/mg protein)

Control	DA: 176.3 ± 6.9	DOPAC: 8.6 ± 0.61
LY5953A (5μM)	DA: 132.0 ± 11.6*	DOPAC: 6.9 ± 0.8
LY5953A (5μM) + 3PPP (5μM)	DA: 124.1 ± 8.1*	DOPAC: 5.9 ± 0.8

(* $p < 0.05$, two-tailed Student 't' test)

evoked overflow of DOPAC as well (to 4.32 ng/mg protein). Although basal and evoked DOPAC were greatly increased the overflow of DA was at about control level. Similarly, preliminary experiments with low doses of 3PPP showed the opposite effect of the higher doses (see above) in that DA evoked overflow was markedly enhanced at S_2 to 3.67 ng/mg protein (n=2) in the presence of 0.1μM ^{3}PPP.

(m) The long-term (non-stimulated) basal overflow of DA and DOPAC

In order to observe the variation of basal overflow over longer periods of time, in the absence of applied depolarizing stimuli, striatal slices were superfused with Krebs solution (as before) for 80 min. Three superfusate samples from between, 10-20, 40-50 and 70-80 min of superfusion were analysed for their DA and DOPAC content, as shown in Figure 3.27.

In the absence of drugs (Figure 3.27), the average DA and DOPAC basal overflow rose from a mean of 0.10 ± 0.01 (range 0.16 to 0.03) ng DA/mg protein/2 min and 0.50 ± 0.02 (range 0.69 to 0.38) ng DOPAC/mg protein/2 min during 10-20 min of superfusion to 0.27 ± 0.05 (range 0.65 to 0.10) ng DA/mg protein/2 min and 0.97 ± 0.10 (range 2.03 to 0.76) ng DOPAC/mg protein/2 min during the 70-80 min of superfusion. At the end of the experiment the DA/DOPAC content of the tissues was found to be 173.2 ± 5.2 and 4.6 ± 0.25 ng/mg protein respectively, and hence, was not different from control.

During preliminary experiments with haloperidol (0.5μM), present from the start of superfusion, none of the above mentioned values appeared altered.

3PPP (5μM) added to the superfusing Krebs solution from the start of superfusion, abolished the increase with time, of the DA and DOPAC basal overflow. The values obtained were as overleaf (n=4).

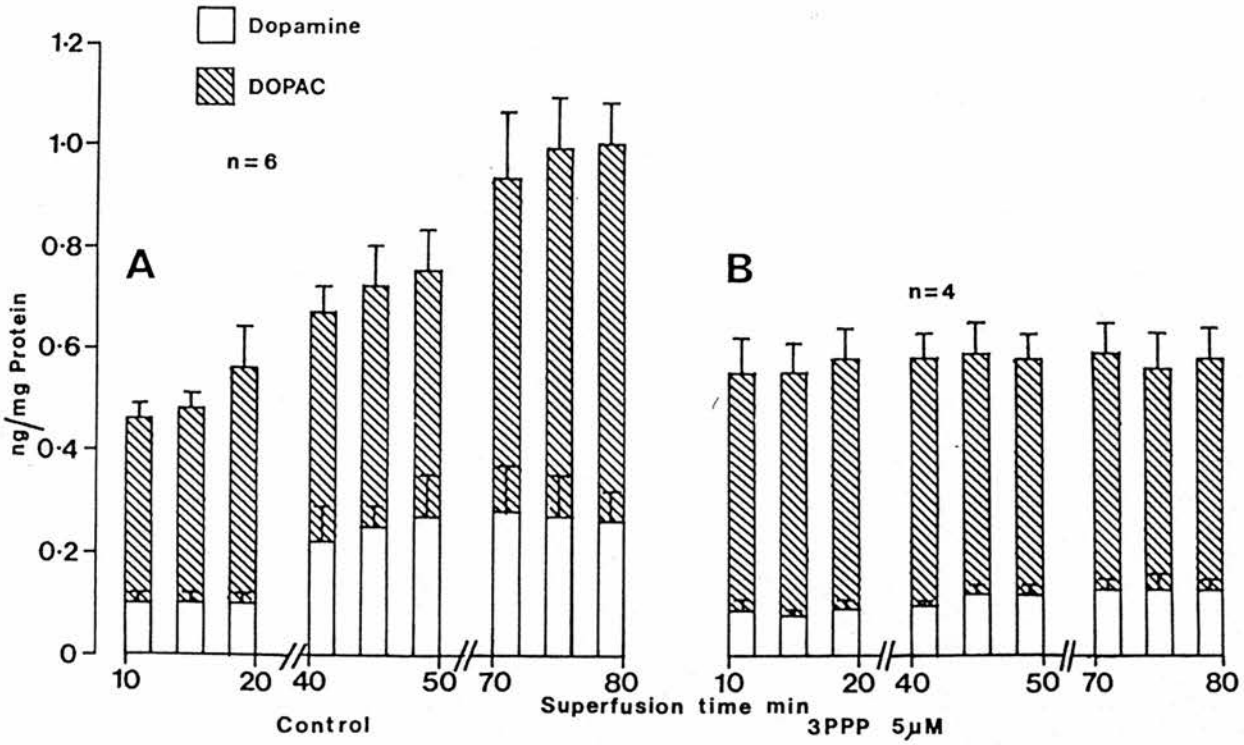


FIGURE 3.27: A. The basal overflow of DA and DOPAC was measured from three superfusate samples between each of 10-20, 40-50 and 70-80 minutes of superfusion. No depolarizing stimuli were applied. As shown above, the basal overflow of both DA and DOPAC rose with time.

B. In the presence of 3PPP (5μM), however, the increase in the basal overflow of DA and DOPAC with time noted above, was abolished.

DA/DOPAC content of the tissues at the end of the experiments (ng/mg protein)

Control (non-stimulated)	DA: 173.2 ± 5.2	DOPAC: 6.0 ± 1.2
3PPP (non-stimulated)	DA: 109.3 ± 2.6*	DOPAC: 3.2 ± 0.4*

(* $p < 0.05$, two-tailed Student 't' test)

Superfusion time (min):	Basal overflow (mean ± s.e.m.) ng/mg protein/2 min 10-20	40-50	70-80
Dopamine	0.09 ± 0.02	0.10 ± 0.01	0.13 ± 0.02
DOPAC	0.55 ± 0.06	0.58 ± 0.05	0.58 ± 0.06

At the end of the experiments in the presence of 3PPP, the DA and DOPAC content of the tissues was found to be significantly lower than in control experiments, i.e. 109.3 ± 1.6 and 3.2 ± 0.4 ng/mg protein, respectively (see Figure 3.27).

(n) Comparison between endogenous DA and ^3H overflow

i) Control:

Striatal slices preincubated with ^3H-DA (4µl of [^3H-7,8]-dopamine, 47 ci/mM) were superfused with oxygenated Krebs and stimulated twice (as above). The superfusate from each 2 min collection was analysed as follows:

1. 20µl was injected into the HPLC-ECD to determine the DA/DOPAC content.
2. 500µl was added to 8 ml of scintillation fluid and its ^3H content analysed by liquid scintillation counting.

The results, calculated as before, show that while the basal overflow of both DA and DOPAC was not different from control, the evoked overflow of DA was markedly enhanced to 2.16 ± 0.47 (range 3.22 to 1.16) ng/mg protein (n=4) at S_1 and 1.78 ± 0.28 (range 2.38 to 1.07) ng/mg protein at S_2, giving an S_2/S_1 ratio of 0.88 ± 0.08 (see Figure 3.28). The evoked overflow of DOPAC did not significantly differ from control levels (i.e. 2.44 ± 0.43 ng/mg protein at S_1 and 2.15 ± 0.44 ng/mg protein at S_2).

FIGURE 3.28: A. The basal and evoked overflow of DA and DOPAC under control conditions, from Figure 3.16.

B. The basal and evoked overflow of DA and DOPAC from tissue preincubated with ^{3}H-DA. While the basal overflow of DA and DOPAC remained unaltered both at S_1 and S_2, the evoked overflow of DA was greatly enhanced. The DOPAC evoked overflow at S_1 and S_2 was not significantly different from control.

C. 3PPP (5μM) added to the superfusing solution of tissue preincubated with ^{3}H-DA at 30 min, enhanced the basal DOPAC overflow while decreasing the evoked overflow of both DA and DOPAC.

D. The evoked overflow of ^{3}H at S_1 was slightly but not significantly higher than that found at S_2, from tissue preincubated with ^{3}H-DA (simultaneous measurement from experiments shown above, B). For basal overflow see text.

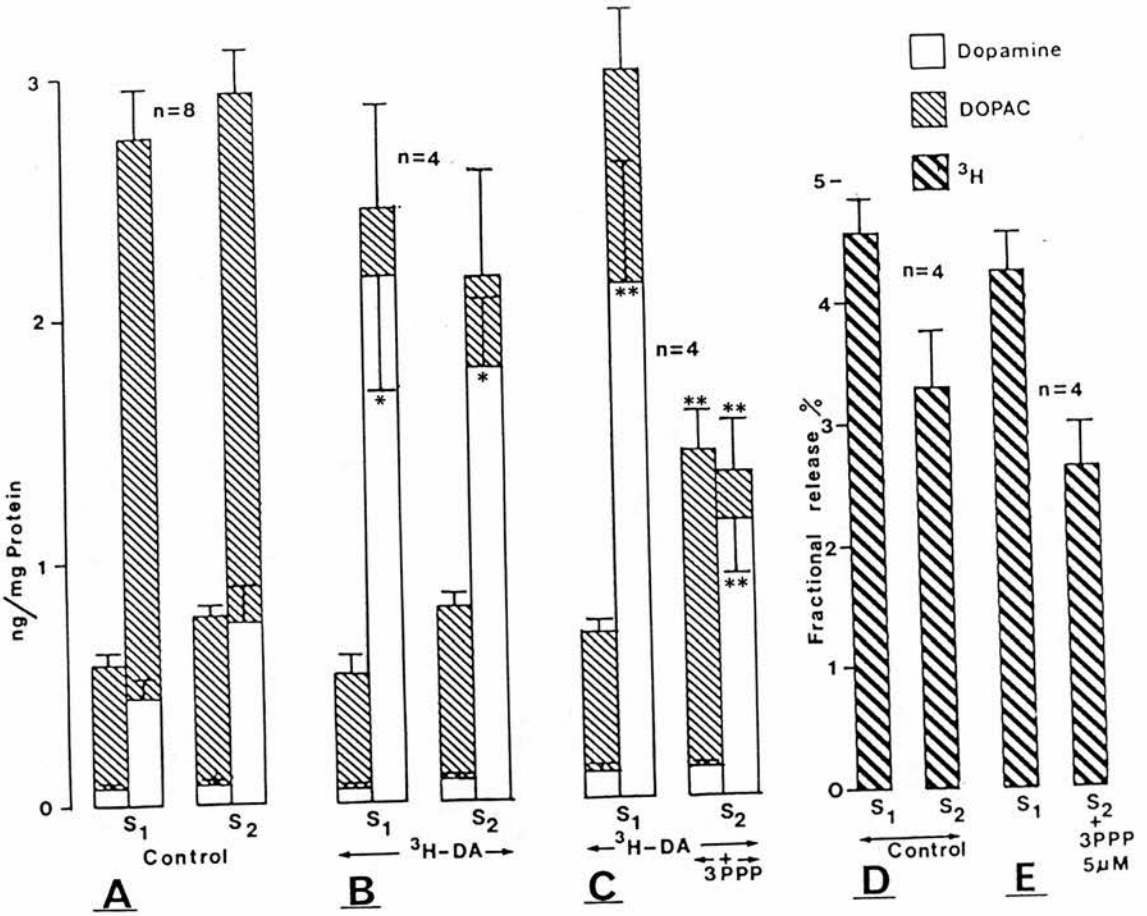
E. The simultaneous measurement of ^{3}H overflow from experiments described above in C, it was found that the evoked overflow of ^{3}H at S_1 was larger than that at S_2, in the presence of 3PPP (5μM). However, the presence of 3PPP did not significantly alter the overflow ^{3}H from that seen under control conditions (D).

DA/DOPAC content of the tissues at the end of the experiments (ng/mg protein)

Control	DA: 176.3 ± 6.9	DOPAC: 8.6 ± 0.61
^{3}H-preincubated	DA: 155.5 ± 6.6	DOPAC: 7.0 ± 0.75
^{3}H-preincubated + 3PPP	DA: 131.2 ± 6.3*	DOPAC: 6.9 ± 0.77

(* $p < 0.05$, two tailed Student 't' test)

(** $p < 0.05$, two-tailed Student 't' test for values significantly different from ^{3}H-preincubation alone)


Dopamine
DOPAC
3H
ng/mg Protein
Fractional release %
n=8
n=4
n=4
n=4
n=4
S1
S2
Control
3H-DA
3PPP
S2 + 3PPP 5µM
A
B
C
D
E

The per cent fractional basal overflow of ^3H at S_1 did not differ from that at S_2 and was found to be 0.64 ± 0.08%. The evoked fractional overflow at S_1 was 4.56 ± 0.29 (range 5.21 to 3.68) % (n=4) with a decrease (not significant) at S_2 to 3.30 ± 0.47 (range 4.36 to 1.79) %.

ii) The effect of uptake inhibition on the evoked overflow of DA and ^3H:

In the presence (from the start of superfusion) of LY5953A (5μM) the basal overflow of DA from tissue preincubated with ^3H-DA was significantly higher both at S_1, i.e. 0.29 ± 0.04 ng/mg protein/2 min and at S_2, i.e. 0.50 ± 0.03 ng/mg protein/2 min, than that found in control tissue (see Figure 3.29). The basal overflow of DOPAC, however, did not differ significantly from that found in presence of 5μM LY5953A in the absence of ^3H-DA preincubation.

The evoked overflow of DA at S_1, in the presence of the uptake inhibitor, from tissue preincubated with ^3H-DA was about 30% higher (i.e. 6.83 ± 0.34, range 7.98 to 5.91, ng/mg protein) than that observed from control tissue in the presence of 5μM LY5953A.

Hence while there was a fourfold difference in the DA overflow, between control tissue and tissue preincubated with ^3H-DA, in the presence of the uptake inhibitor this difference was reduced to 30%.

At S_2, however, the evoked overflow of DA under these conditions was more than 20% lower than that seen from control tissue in the presence of 5μM LY5953A (see Figure 3.29). The evoked overflow of DOPAC at S_2 was also reduced to 1.52 ± 0.34 ng/mg protein, although DOPAC overflow at S_1 remained unaltered, giving an S_2/S_1 ratio of 0.51 ± 0.09.

FIGURE 3.29: A. The basal and evoked overflow of DA and DOPAC in the presence of 5μM LY5953A, from control tissue (from Figure 3.26).

B. The basal and evoked overflow of DA and DOPAC in the presence of 5μM LY5953A, from tissue preincubated with ^{3}H-DA. The evoked overflow of DA at S_1 was significantly higher than control, while that at S_2 was significantly lower than control. The evoked overflow of DOPAC at S_2 also appeared to be lower than control.

C. The addition of haloperidol (0.5μM) (Halop.), to tissue preincubated with ^{3}H-DA and superfused in the presence of 5μM LY5953A significantly enhanced the evoked overflow of DA and DOPAC at S_2 from that seen in the presence of LY5953A alone (B, ** - values significantly different, $p < 0.05$ two-tailed Student 't' test).

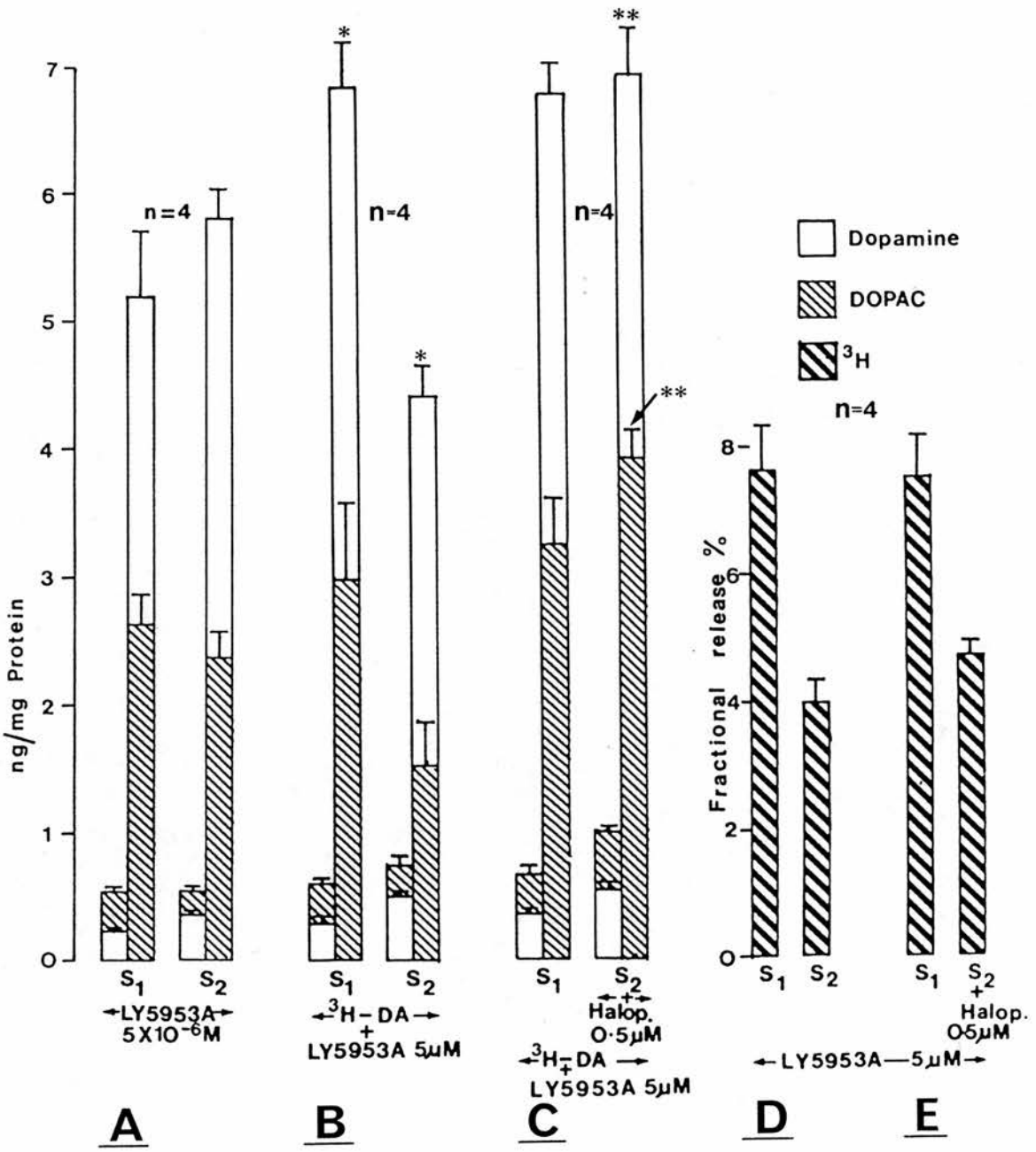
D. The evoked overflow of ^{3}H, at S_1, in the presence of LY5953A (5μM) was significantly higher than control (see Figure 3.28); however, despite the continued presence of LY5953A, the evoked overflow of ^{3}H at S_2 did not differ from control.

E. Haloperidol (0.5μM) (Halop.) did not alter the evoked overflow of ^{3}H at S_2 (in the presence of LY5953A, 5μM).

DA/DOPAC content of the tissue at the end of the experiments (ng/mg protein)

LY5953A (5μM)	DA: 132.0 ± 11.6	DOPAC: 6.9 ± 0.8
^{3}H-DA + LY5953A (5μM)	DA: 124.0 ± 8.1	DOPAC: 5.9 ± 0.8
^{3}H-DA + LY5953A (5μM) + Halop. (0.5μM)	DA: 165.0 ± 3.7*	DOPAC: 10.8 ± 0.6*

(* $p < 0.05$, two-tailed Student 't' test, difference from respective control).


ng/mg Protein
n=4
n=4
n=4
n=4
Dopamine
DOPAC
^{3}H
Fractional release %
S1
S2
←LY5953A→
5X10^{-6}M
←^{3}H-DA→
+
LY5953A 5μM
Halop.
0·5μM
←LY5953A—5μM→
A
B
C
D
E

The basal overflow of ^3H (fractional overflow x 100) at S_1 (0.93 ± 0.09%) and S_2 (1.3 ± 0.1%) was significantly higher than in the absence of LY5953A. The evoked overflow of ^3H in the presence of the uptake inhibitor at S_1 was significantly higher than control, i.e. 7.75 ± 0.59%; this ratio of evoked ^3H in the presence and absence of the uptake inhibitor at S_1 was 1.7, compared to 3.2 for authentic DA during the same experiments (the same ratio in the absence of ^3H-DA preincubation was 11.8, see Figure 3.29 and Figure 3.16).

The evoked overflow of ^3H at S_2 in the presence of an uptake inhibitor did not differ significantly from control, during the same experiment, however, DA overflow was still 2.5-fold higher in the presence than in the absence of the uptake inhibitor (see Figures 3.29 and 3.28).

iii) The effect of 3PPP on the evoked overflow of DA and ^3H

Addition of 3PPP (5μM) before S_2, to tissue preincubated with ^3H-DA enhanced the basal overflow of DOPAC at S_2 to 1.42 ± 0.16 (range 2.01 to 0.84) ng/mg protein/2 min while not affecting the DA basal overflow. The evoked overflow of both DA (1.13 ± 0.22, range 1.62 to 0.55 ng/mg protein) and DOPAC (1.33 ± 0.21, range 2.01 to 0.84, ng/mg protein) at S_2 were reduced (see Figure 3.28, cf. Figure 3.25). The DA content of the tissue at the end of the experiment was 136.0 ± 8.6 ng/mg protein; although lower than control values, it did not significantly differ from that found in the presence of ^3H only. The evoked overflow of ^3H at S_2, in the presence of 3PPP, was slightly (but not significantly) lower than control levels (see Figure 3.28). The basal overflow of ^3H remained unaffected.

iv) The effect of haloperidol on the evoked DA and ^3H overflow in the presence of an uptake inhibitor:

Preliminary experiments with 3PPP (5μM) added before S_2 in the presence of 5μM LY5953A showed no difference in the basal or evoked overflow of either DA or DOPAC. 0.5μM haloperidol added 30 min after the onset of superfusion, in the presence of 5μM LY5953A, enhanced the basal overflow of DOPAC to 0.99 ± 0.05 (range 1.09 to 0.89) ng/mg protein/2 min while not affecting DA basal overflow. The evoked overflow of DA was increased to 7.12 ± 0.36 (range 8.22 to 6.2) ng/mg protein (n=4) as was the evoked overflow of DOPAC, i.e. 3.92 ± 0.23 (range 4.38 to 3.20) ng/mg protein.

Both the DA and DOPAC content of the tissue at the end of the experiment were significantly higher than those found during experiments without haloperidol (see Figure 3.29).

Although the basal and evoked overflow of ^3H at S_2 were higher in the presence of haloperidol (1.51 ± 0.09% and 4.75 ± 0.20%, respectively), they were not significantly different from values obtained in the presence of only uptake inhibitor (i.e. basal overflow, 1.31 ± 0.1% and evoked overflow, 4.05 ± 0.30%).

3.4 Discussion

The results presented in Chapter 3.1 confirm that the DA/DOPAC measured by a direct analysis of tissue extracts or superfusate samples use mostly authentic DA/DOPAC, as simultaneous measurements by GCMS and under different chromatographic conditions during HPLC-ECD, yielded similar results. The evoked overflow of ^{3}H-DA, previously preincubated with the tissue, co-eluted with endogenous DA and the peaks eluting at the retention time of authentic DA/DOPAC from superfusate samples were alumina extractable. This conclusion was further justified by the finding that the increases in DA/DOPAC in superfusate samples during depolarizing stimuli were Ca^{++} dependent (electrically evoked overflow of DA/DOPAC was also TTX dependent). While the DA/DOPAC overflow remained unaltered after KA lesions of the striatum, it was completely abolished by 6-OHDA lesions. Pargyline abolished the DOPAC peak selectively, while AMPT inhibited the overflow of both DA and DOPAC.

Since the development of HPLC-ECD in the early 1970's (Keller *et al.*, 1976; Kissinger *et al.*, 1973), it has already come to be routinely used for measuring catecholamines, indolamines and their metabolites from tissue and plasma samples (for recent reviews, see Kissinger *et al.*, 1981; Mefford, 1981). Mayer and Shoup (1982) have recently suggested that the ratio of the peak height of a compound oxidised at different working potentials may be used as a 'fingerprint' for that compound to enhance the selectivity of HPLC-ECD.

The use of HPLC-ECD to detect the overflow of endogenous catecholaminergic neurotransmitters *in vitro* was first described by Plotsky *et al.*, 1977. However, this and subsequent experiments measuring endogenous DA overflow from striatal tissue (Nahorski and

Strupish, 1981; Bennet *et al.*, 1981) were conducted on tissue "incubated" in Krebs solution and not superfused (present results), making a direct comparison of values difficult. Despite these difficulties, however, the basal overflow of DA found by Bennet *et al.*, (1981) and here are almost identical (0.08 ng/mg protein/2 min). 50mM K^+ during the present study induced roughly the same amount of DA overflow as 55mM K^+ as described by Bennet *et al.*, (1981).

Using similar techniques, the *in vitro* overflow of endogenous 5-HT from brain slice preparations (Bennet *et al.*, 1980; Irons *et al.*, 1982; C.A. Marsden, personal communication) and from fore-brain synaptosomes (Collard *et al.*, 1981) have also been described. Although these authors found a higher (about sixfold) basal overflow of 5-HIAA compared to the basal overflow of 5-HT, no increases in the overflow of 5-HIAA during the high K^+ induced overflow of 5-HT were found.

Little data on the overflow of endogenous DOPAC *in vitro* is available, however, recent investigations by Ungerstedt and co-workers (personal communication; Ungerstedt *et al.*, 1982; Bennet *et al.*, 1982) on the *in vivo* overflow of DA and DOPAC mainly from the striatum (intra cerebral dialysis combined with HPLC-ECD) shows a similar pattern to that found in the present investigations during electrical stimulation (i.e. a large overflow of DOPAC accompanied by more than an order of magnitude smaller overflow of DA) despite differences in methodology.

In vivo determination of endogenous DA (see Glowinski *et al.*, 1979) and its metabolite overflow (HVA, Portig and Vogt, 1969) also showed similar trends to those found during the present thesis.

The site of origin of DOPAC

A mainly presynaptic origin of DOPAC (for early evidence see review by Sharman, 1973) has been suggested since indirectly acting sympathomimetics drugs (e.g. reserpine) increase only the overflow of DOPAC (Cubeddu *et al.*, 1979) and the relatively high basal overflow of ^{3}H-DOPAC, from tissue preincubated with ^{3}H-DA (i.e. 68% of the fractional release), is unaffected by uptake inhibitors (Zumstein *et al.*, 1981) but is inhibited by amezinium which inhibits MAO inside nerve terminals after being taken up (Steppler and Starke, 1980). This is supported by the present findings that inhibition of impulse flow by TTX, or DA release by Ca^{++} free media does not affect basal overflow of DOPAC (i.e. no inherent depolarizing mechanisms appear to be necessary), and the basal and evoked overflow of DOPAC are largely unaffected under conditions of uptake inhibition, despite marked increases in DA overflow.

Therefore, DOPAC which appears to leak out of the presynaptic terminal readily (Trendelenberg *et al.*, 1980) may reflect changes in DA formation more closely than in DA release (Westerink and Korf, 1976). Hence, the increase in tissue levels of DOPAC *in vivo* after impulse inhibition reflects an increased synthesis of DA (Di Giulio *et al.*, 1978; Broxterman *et al.*, 1980).

During the present experiments, this important distinction, that the DOPAC overflow reflects mainly alterations of the rate of formation of DA and not primarily in its rate of utilization, was suggested by the findings that:

1. During the K^{+} induced overflow of DA (which is not affected by the uptake inhibitor, nomifensine, and so appears to approximate DA release) there is a marked increase in DOPAC formation, suggesting

an increase in DA synthesis. In support of this view, Schwarz *et al.* (1980) showed an increase in the rate of synthesis of ^{3}H-L-DOPA (from ^{3}H-tyrosine) during incubation of striatal slices with 55mM K^{+} media.

Interestingly, while DA overflow increases dramatically with increasing K^{+} concentration during K^{+} stimulation (about 15-fold between 15 and 50mM K^{+}), there is a smaller increase in DOPAC overflow (about threefold), suggesting a different dose/response relationship. This implies that the formation of DOPAC does not depend entirely on the release (utilization) of DA, but depends on the synthesis of DA since DOPAC is derived from DA; as is suggested in the finding that DOPAC formation during electrical and K^{+} stimulation is the same while there is a large difference in DA overflow.

2. Despite a marked increase in the basal and electrically evoked overflow of DA in the presence of an uptake inhibitor, the basal and evoked overflow of DOPAC remained largely unaltered, as mentioned before. It was not possible, however, to determine what part of the overflow of DOPAC represents the deamination of the recaptured DA during the present experiments.

The implication of the lack of effect of uptake inhibitors on the electrically evoked overflow of endogenous DOPAC at S_1, and the small reduction at S_2, is either: a) little recaptured DA is deaminated, or b) since Zumstein *et al.*,(1981) have shown that some of the released ^{3}H-DA (electrically evoked), is taken up by nerve terminals and deaminated resulting in an evoked overflow of ^{3}H-DOPAC, implies that the decrease in endogenous DOPAC overflow expected after DA uptake inhibition, may be compensated for by an increased DOPAC formation from newly-synthesised DA. Thus, the evoked DOPAC

overflow may reflect the increased DA synthesis associated with stimulation.

3. Ten minutes after the administration of AMPT to striatal slices, although inhibition of TH may have been incomplete (Doteuchi *et al.*, 1974), the basal overflow of DOPAC was reduced by about 40%, and on stimulation the evoked overflow of DOPAC was only about 8% of control values, even though DA overflow was reduced by only about 50% (see later). Twenty-six minutes later, the basal overflow of DOPAC was reduced by 70% and the evoked overflow almost abolished (at S_2), in spite of the fact that at the end of the experiment the tissue content of DA was the same as values found before the start of superfusion of control experiments (i.e. about half the amount present at the end of control experiments).

After long-term inhibition of TH with AMPT (2 h after *in vivo* administration) while the tissue content of DA is reduced (40% of control start of experiment values), no overflow of either DA or DOPAC could be detected. This implies that most of the DOPAC overflow is derived from the 'newly synthesized-releasable pool' of DA or the 'cytoplasmic pool' described by Broxterman *et al.* (1979).

These suggestions are in line with the findings of Groppetti *et al.*, (1977) that the specific activity of DOPAC rises faster than that of DA after intra-ventricular administration of ^{3}H-tyrosine.

4. The basal overflow of DA and DOPAC increased with time in the absence of applied depolarizing stimuli.

Interestingly, the increase and rate of increase of DOPAC overflow found *in vitro*, are similar to those found *in vivo* by Broxterman *et al.*, (1980) after HA-966 (a γ-butyrolactone, GBL, like drug)

administration. The DA content of the tissue at the end of *in vitro* non-stimulated experiments was also similar to that described by the above mentioned authors *in vivo*.

Hence it appears that non-stimulated striatal slices *in vitro*, behave similar to the striatum *in vivo* under conditions of impulse flow inhibition.

The D_2 agonist 3PPP (Hjorth *et al.*, 1981; Watling and Williams, 1982) abolished the *in vitro* increase in DOPAC overflow seen in the absence of applied depolarizing stimuli, suggesting that in the absence of DA release, dopaminergic presynaptic receptors which decrease DA turnover, are not stimulated.

Similarly, Walters and Roth (1976) showed that the *in vivo* increase in DA turnover after GBL administration could be abolished by dopaminergic agonists, but remains unaffected by dopaminergic antagonists (Nowycky and Roth, 1978; see page 32).

Dopamine overflow

The basal overflow of DA was found to be very low under control conditions (less than 0.1% of tissue DA content). In the absence of applied depolarizing stimuli, an increased basal overflow of DA with time was found during the present study.

Similarly, Cheramy *et al.*,(1977),while examining the overflow of ^{3}H-DA newly synthesized from ^{3}H-tyrosine *in vivo* after impulse flow inhibition with γ-hydroxybutyrate, found an increasing basal overflow of ^{3}H-DA in the absence of impulse flow. The *in vitro* increase of DA basal overflow was found to be inhibited with 3PPP during the present study.

The large increase in the electrically evoked overflow of DA in the presence of 5µM LY5953A (a specific DA uptake blocker, Wedley

et al., 1978), suggested that up to 90% of the released DA is taken back up by nerve terminals.

In a detailed study of the chemical composition of the released ^{3}H (^{3}H-DA preincubation) from the rabbit caudate nucleus, Zumstein *et al.*,(1981) showed that the increase in ^{3}H-DA overflow after nomifensine (1μM) was completely obscured by the ^{3}H-DOPAC overflow when only total ^{3}H overflow was examined.

While the electrically evoked overflow of DA was increased by nomifensine (1μM) during the present experiments, the K^{+} evoked overflow of DA was unaffected. Raiteri *et al.*,(1978) also found no effect of 1μM nomifensine on the 55mM K^{+} evoked overflow of ^{3}H-DA (which consists mainly of ^{3}H-DA, De Langen *et al.*, 1979; Cubeddu *et al.*, 1979), Dambiec and Cohen (1981) reported a decreased K^{+} evoked overflow of ^{3}H (alumina extract) in the presence of uptake inhibitors at higher doses (10μM).

A larger overflow of DA during K^{+} compared to electrical depolarization may be indicative of the presence of a high affinity uptake system, which is inhibited by high K^{+} concentrations.

While changes in current strength were not tested, the evoked overflow of DA and DOPAC was Ca^{++} and TTX dependent at 8-10mA. Aceves and Cuello (1981) showed that while the overflow of ^{3}H was Ca^{++} and TTX dependent up to 9mA stimulation amplitude, the larger current strengths commonly used (20mA) resulted in a Ca^{++} and TTX independent overflow of ^{3}H.

The overflow of DA increased with increasing frequency (constant number of pulses) to a maximum around 20Hz, similar values have been reported by various authors for a maximal increase in tissue metabolite levels after *in vivo* stimulation of the median fore-brain bundle (Korf *et al.*, 1976; Roth *et al.*, 1976).

Altering the duration the stimulus was applied for, at a constant frequency (20Hz), showed that the overflow of DA per impulse declined with increasing number of applied pulses. Bennet and Middleton (1975) found that a similar train length dependent depression of NA release in the peripheral nervous system appeared to be inhibited by α-antagonists. It would be interesting to see the effect of dopaminergic antagonists and agonist on the depression of DA overflow from striatal slices.

Compartmentation of intraneuronal dopamine

Interestingly, even at the highest concentrations tried (50mM), K^+ depolarization resulted in the overflow (release) of only about 30% of the tissue content of DA, suggesting that not all the DA present in the tissue is available for release.

Further support for the compartmentation of intraneuronal DA into stored and releasable pools (see introduction, 3.3), was obtained from a study of the overflow of DA after TH inhibition. Two hours after *in vivo* administration of AMPT, although more than 40% of tissue DA was retained, neither basal nor evoked overflow of DA was detectable despite the presence of 5μM LY5953A. Short term *in vitro* inhibition of TH with AMPT significantly inhibited the electrically induced overflow of DA (despite the possibility of uptake inhibition of DA by the AMPT, and incomplete inhibition of TH at S_1 as suggested earlier).

These findings support the suggestion of Glowinski (1975), Groppetti *et al.*,(1977) and Shore *et al.*,(1980) of a preferential release of newly synthesized DA.

The effects of presynaptic receptors on the DA and DOPAC overflow

(1) Muscarinic agonists:

The present investigations show that cholinergic agents - directing acting muscarinic agonists or acetylcholine esterase inhibitors (elevating the levels of evoked endogenous ACh overflow), markedly potentiated the K^+ and electrically evoked DA and DOPAC overflow. These effects could be inhibited by atropine, but not by tubocurarine or gallamine, indicating the involvement of muscarinic type cholinergic receptors. The effect of the muscarinic agents persisted in the presence of the DA uptake inhibitor, LY5953A, suggesting that muscarinic presynaptic receptor stimulation increases the release and rate of synthesis of DA.

The effects of acetylcholine esterase inhibitors was unaffected by KA lesions of the striatum, indicating the possible involvement of cholinergic afferents to the striatum (Smike and Saelens, 1977), or that not all the cholinergic cell bodies within the striatum are destroyed by KA (Kramer *et al.*, 1979).

Since all the evidence presented above was obtained from striatal slices containing nerve terminals in the absence of cell bodies, a presynaptic site of action is implied. Although the existence of a 'short loop feedback' by cholinergic interneurons was not completely ruled out, its absence is suggested by the persistence of the muscarinic presynaptic effects after KA lesions of the striatum, and in synaptosomal preparations, as was recently found by Marchi *et al.*,(1982).

The present findings support those of Lloyd and Bartholini (1975), who, using a push-pull cannula *in vivo*, showed that oxo-tremorine markedly potentiated the overflow of endogenous DA. Although Giorguieff *et al.*,(1977b) reported a muscarinic presynaptic

receptor mediated increase in the basal overflow of ^{3}H-DA (newly-synthesized from ^{3}H-tyrosine), interpretation of their results is complicated by the fact that an increased specific activity of the overflow of DA during a constant basal release was not ruled out (see introduction, page 34).

The influence of muscarinic agents on the evoked overflow of ^{3}H-DA (after preincubation) is more controversial. While Westfall (1974a,b,c) and De Belleroche and Bradford (1978) reported an inhibitory influence of muscarinic agonists on ^{3}H-DA overflow, the work of Marchi *et al.* (1982) on striatal synaptosomes, and that of De Belleroche and Gardinier (1982) on nucleus accumbens slices and Perkins and Westfall (1979) on hypothalamic slices, indicates a small (30-40%) facilitation of evoked ^{3}H overflow with muscarinic agonists.

During the present thesis, the simultaneous determination of ^{3}H and endogenous DA/DOPAC overflow, showed that while the endogenous DA/DOPAC evoked overflow was facilitated (>80%) by muscarinic agonists, there was only a small (about 35%) simultaneous facilitation of ^{3}H overflow.

Hence, the implication is that muscarinic agonists facilitate the synthesis and utilization of the newly-synthesized DA.

A muscarinic agonist induced increase of DA turnover has been reported by several authors. Paalzov and Paalzov (1975) found an increased AMPT-induced disappearance of cerebral DA. Muscarinic agonists have also been shown to raise dopamine metabolite (HVA and DOPAC) levels (Laverty and Sharman, 1965; Nose and Takemoto, 1974) in the striatum, while O'Keeffe *et al.*,(1970) found a decrease in striatal HVA after atropine. Interestingly, HVA has been shown to be derived mainly (80%) from DOPAC (Westerink and Spaan, 1982a)

and so most likely reflects changes in the rate of DA synthesis. 3-MT on the other hand, is thought to be a better index of DA overflow (Westerink and Spaan, 1982a,b; Westerink and Korf, 1976; Kehr, 1976).

Javoy *et al.*,(1975) showed an increased L-DOPA formation *in vivo* after oxotremorine administration (Westerink *et al.*, 1982c). The same authors also reported that although the specific activity of tissue DA (^{3}H-tyrosine administered I.V.) was also enhanced, the total DA content of the striatal tissue remained unaltered, suggesting a muscarinic receptor mediated facilitation of DA synthesis and utilization simulationeously.

Nicotine enhanced the basal overflow of both DA and DOPAC confirming the findings of Giorguieff *et al.*,(1976, 1979a). Nicotine did not, however, have any significant effect on the evoked overflow of either DA or DOPAC during the current investigations.

(2) Dopaminergic presynaptic receptors:

The involvement of dopaminergic presynaptic receptors regulating the rate of synthesis of DA, in the absence of depolarizing stimuli has been discussed above (see also introduction, page 31, and Table 1).

The present investigations showed that the dopaminergic agonist, 3PPP, decreased the evoked overflow of both DA and DOPAC, while haloperidol had the opposite effect. The effects of these dopaminergic agents persisted in the presence of the uptake inhibitor, LY5953A, indicating that release rather than overflow of DA was altered. Alterations of the overflow of DOPAC suggest an inhibitory effect of dopaminergic presynaptic receptors on the rate of synthesis of DA during stimulation.

The controversy in the literature about the actions of neuroleptics on the overflow of ^{3}H-DA remains unresolved; Lehmann *et al.*,(1981) have recently ruled out species difference (rabbit, cat, rat) and the inhibitory effects of ascorbic acid on dopamine receptor mediated actions (Thomas and Zemp, 1977; Kayaalp and Neff, 1980) as possible causes of the differences of opinion expresses in the literature (see Table 1). As suggested in the introduction (page 33), the most likely explanation for the lack of effect or inhibition of ^{3}H-DA release with neuroleptics is the presence of MAOI and perhaps small differences in experimental protocol. This view is supported by the finding of Zumstein *et al.*,(1981) that the inhibitory effects of apomorphine on the overflow of ^{3}H are abolished by pargyline.

After the original description of ^{3}H-DA overflow inhibiting dopaminergic presynaptic receptors by Farnebo and Hamberger (1971), further supportive evidence came from *in vivo* studies of endogenous DA overflow (Lloyd and Bartholini, 1975) and *in vitro* studies on the overflow of endogenous (Plotsky *et al.*, 1977) and newly-synthesized (Westfall *et al.*, 1976) DA. Starke *et al.*,(1978) subsequently confirmed the findings of Farnebo and Hamberger (1971) and showed a presynaptic dopamine receptor mediated depression of ^{3}H-DA overflow *in vitro*, using dopaminergic agonists and antagonists (as have several authors since then, see Table 1 for references).

During the above mentioned studies, the neuroleptic induced facilitation of ^{3}H-DA release is usually found to be no more than about 50-60% above control levels (Starke *et al.*, 1978; Miller and Friedhoff, 1979), the facilitation of endogenous or newly-synthesized DA with neuroleptics is, however, reported to be much higher, i.e. about 150-300% (see above for references, present study). This suggests

that dopaminergic antagonists facilitate the release mainly of newly synthesized DA. During the present study, this suggestion is supported by the finding that although there was a marked facilitatory effect of haloperidol on endogenous DA and DOPAC overflow and a significant inhibitory effect of 3PPP, simultaneous determination of ^{3}H overflow failed to follow these changes accurately.

Comparison between endogenous DA/DOPAC and ^{3}H overflow

During experiments with tissue preincubated with ^{3}H-DA, the overflow of endogenous DA and DOPAC (as measured by HPLC-ECD) differed from that found during control experiments in several ways:

1. There was a much greater electrically evoked overflow of endogenous DA from tissue preincubated with ^{3}H-DA compared to control tissue. When uptake was inhibited (high K^{+} or electrical stimulation in the presence of 5μM LY5953A), however, the overflow of endogenous DA from control or ^{3}H-DA preincubated tissue did not markedly differ. Initial studies measuring the overflow of DA from tissue preincubated with 'cold' DA (results not presented) indicated that part of the apparent uptake inhibition with ^{3}H-DA may be due to an inhibitory action of exogenous DA on uptake. However, the possibility of uptake inhibition by ethanol or acetic acid, in which the ^{3}H-DA is supplied, or the presence of ^{3}H, was not ruled out. The overflow of exogenous DA from sites other than DA nerve terminal may also have contributed to this apparent uptake inhibition.
2. The evoked overflow of DA from control slices in the presence of LY5953A was roughly the same at both S_1 and S_2 (S_2/S_1 = 1.12). However, there was a significantly lower release at S_2, from striata preincubated in with ^{3}H-DA and superfused in the presence of 5μM LY5953A (S_2/S_1 = 0.65), a similar reduction of DOPAC overflow was also found at S_2, which was not seen in control striatal slices.

Persistence of the inhibitory actions of 3PPP on DA/DOPAC overflow in control but not ^{3}H-DA preincubated slices (in the presence of LY5953A), and the effectiveness of haloperidol in facilitating the overflow of DA from both tissues under the same conditions, suggests that part of the inhibition of DA/DOPAC at S_2 may have been due to an action of ^{3}H-DA at the presynaptic dopaminergic receptors.

3. Although LY5953A enhanced the overflow of ^{3}H at S_1 by about 80% (c.f. about a tenfold increase of endogenous DA from control tissue), the overflow of ^{3}H at S_2 was not significantly different from control (simultaneous determination of endogenous DA, showed it to be at least 250% higher than control at this stage). This small S_2/S_1 ratio of ^{3}H overflow was unaffected by haloperidol, although as noted above, the S_2/S_1 ratio for endogenous DA, measured simultaneously increased from 0.65 to 1.05 in the presence of haloperidol.

Hence the decreased overflow of ^{3}H at S_2 in the presence of LY5953A may have been due to a depletion of the label from the releasable pool of DA, whereas, as suggested above, the concomitant decrease of endogenous DA may have been due to a stimulation of presynaptic dopaminergic receptors by the added ^{3}H-DA.

DA content of striatal tissue slices:

From an estimation of the total amount of DA and DOPAC overflow into the superfusing Krebs solution, during control electrical stimulations, and the difference between the DA content of the tissue at the start and at the end of the experiments, the value for the total amount of *in vitro* synthesis and utilization of DA per hour was found to be approximately 60nM/g wet weight/hr (assuming that 1 mg protein = 10 mg wet weight). This, however, should not be compared to the

values of DA turnover obtained *in vivo* (Sharman, 1981) as numerous conditions are different, mostly that *in vivo* an ongoing impulse flow from the nigra probably greatly modifies the turnover of DA in the nigro-striatal nerve terminals, whereas *in vitro*, only two 30 sec, 20Hz trains of pulses were applied. The important point, neverthless, is that *in vitro* more than 60% of this 'rate of synthesis' measure actually relates to an increased tissue content of DA; the actual 'utilization' of DA was only about 24nM/g wet weight/hr (most of which was DOPAC overflow and only about 10% comprises DA overflow).

This increase of the tissue content of DA is not seen *in vivo* even after electrical stimulation of the median forebrain bundle (Korf *et al.*, 1976). The findings of Shore *et al.*,(1976, 1979) (see McMillen *et al.*, 1980) on the non-amphetamine class central stimulant amfonelic acid, and its interactions with haloperidol and AMPT, may help to explain the *in vitro* increase in the tissue content of DA. The above mentioned authors have suggested that presynaptic dopaminergic receptors can alter the rate of exchange of DA between the 'newly-synthesised, releasable' and 'storage' pools. This, as suggested earlier, is also implied by the increased depletion of DA by drugs like haloperidol, after synthesis inhibition with AMPT, given the assumption of a preferential release of newly-synthesised DA.

The hypothesis, therefore, is that presynaptic dopaminergic receptors on stimulation decrease not only the synthesis and release of DA, but in addition to this, also decrease the rate of exchange of DA between the storage and releasable pools.

Given the above mentioned hypothesis, and assuming that because of the high rate of flow of the superfusing solution *in vitro*, pre-synaptic receptors are only stimulated during or immediately after the

evoked release of neurotransmitters. The implication, therefore, is that the *in vitro* increase of the DAcontent of the tissues after electrical stimulation (as was found in the absence of stimulation) is due to the lack of adequate stimulation of presynaptic receptors which inhibit the transfer of newly synthesised DA to the storage pool. Hence, in the absence of depolarization, despite an uneffected initial basal overflow of DOPAC, the DA content of striatal tissue remains largely unaltered in the presence of 3PPP but increases about twofold in its absence. Similarly, during electrical stimulation in the presence of 3PPP, while the estimated total DOPAC overflow was not much lower than during control experiments (>5%, despite the 30% decrease of evoked DOPAC overflow at S_2), the actual DAcontent of the tissue fell by over 30% in the presence of 3PPP. This implies that less newly-synthesised DA was stored, and perhaps more deaminated as indicated by the higher basal overflow of DOPAC in the presence of 3PPP before S_2. It may then be suggested that the higher basal and evoked overflow of DOPAC with haloperidol reflects an increased synthesis and utilization of DA from both the releasable and storage pools, resulting in a reduced tissue content of DA.

The effect of muscarinic agents supports this tentative suggestion with the finding that despite an increased evoked overflow of DOPAC, implying an increased synthesis of DA, the tissue content of DA fell by over 30%. This suggests that muscarinic agents enhance the synthesis and utilization of newly-synthesized DA but inhibit the translocation of DA between the storage and releasable pools. Hence, while an increased accumulation of L-DOPA after oxotremorine has been demonstrated (Javoy *et al.*, 1975; Westerink *et al.*, 1982c), an increased DA depletion after AMPT by muscarinic agents was not found by some authors (Paalzov and Paalzov, 1975).

This rather involved interpretation is tentatively suggested, and in order to gain more information on the subject, more detailed experiments are proposed. A study of the specific activity of DA/DOPAC overflow and tissue content, during continuous superfusion with ^{14}C-tyrosine may provide further insight into the role of presynaptic receptors on the release and storage of newly-synthesized DA and the utilization of preformed stored DA.

CHAPTER IV

The overflow of dopamine from other dopamine rich tissue

4.1 Overflow of endogenous DA from median eminence (ME) tissue *in vitro*

A) Results

Four median eminence tissue pieces (ME - approximate total wet weight, 3.5 mg), dissected as described in the methods section (page 57) were superfused with oxygenated Krebs solution at 37°C, at a flow rate of approximately 150µl/min. Electrical stimulations (20Hz, 8-10mA, 30 sec) were carried out as before.

The basal overflow of DA and DOPAC was generally found to be below detectable levels (<0.15 ng/4ME/2 min). Electrical stimulation resulted in a rapid increase in DA overflow (see Figure 4.1), which declined to basal levels within 2 minutes. Usually no evoked overflow of metabolites was detectable (see Figure 4.2). The overflow of DA at S_1 (0.33 ± 0.06, range 0.51 to 0.22 ngDA/4ME) did not differ from that at S_2 (0.33 ± 0.07, range 0.56 to 0.22 ngDA/ME). A two-minute pulse of 45mM K^+ also resulted in approximately the same evoked overflow of DA as seen during electrical stimulation (see Figure 4.1).

The tissue content at the end of control experiments with two electrical stimulations was found to be 6.11 ± 0.38 ng/ME, and although generally lower, was not significantly different from values of DA content of ME immediately after dissection (7.85 ± 0.63 ng/ME).

Addition of the dopaminergic uptake inhibitor nomifensine (1µM), 30 min after the onset of superfusion, enhanced the evoked overflow of DA at S_2 by about 50% to 0.53 ± 0.05 (range 0.61 to 0.36) ngDA/ME (see Figure 4.3).

Addition of oxotremorine (10µm) or prolactin (0.5µg/ml) did not alter the evoked overflow of DA at S_2 (see Figure 4.4).

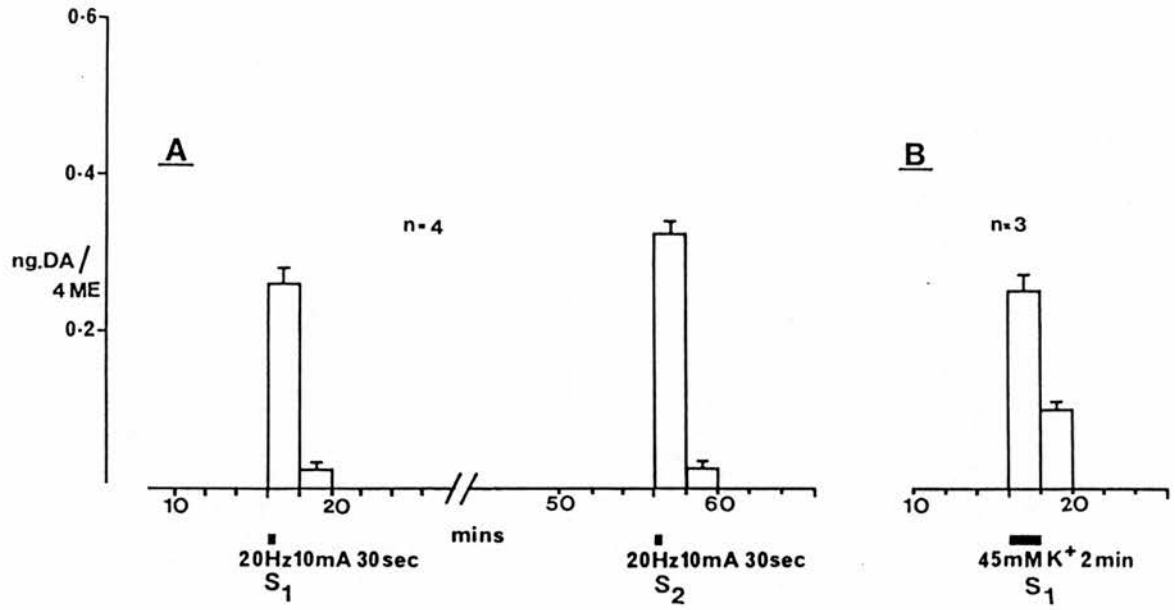


FIGURE 4.1: A. 4 median eminence (ME) pieces superfused with oxygenated Krebs solution (approximately 150µl/min) were stimulated twice at 16 min (S_1) and 56 min (S_2) of superfusion, two minute superfusate sample collections were analysed for their catecholamine content by HPLC-ECD (see Figure 4.2). The basal overflow of DA was generally below detectable levels. During stimulation, however, there was a marked and rapid increase in DA overflow as shown above. Electrical stimulation at S_1 and S_2, and 45mM K^+ stimulation (2 minute pulse) induced the same overflow of DA.

Tissue DA content (ng/ME)

Freshly dissected tissue	DA: 7.85 ± 0.63
Tissue from end of control experiments (A)	DA: 6.11 ± 0.38 N.S.

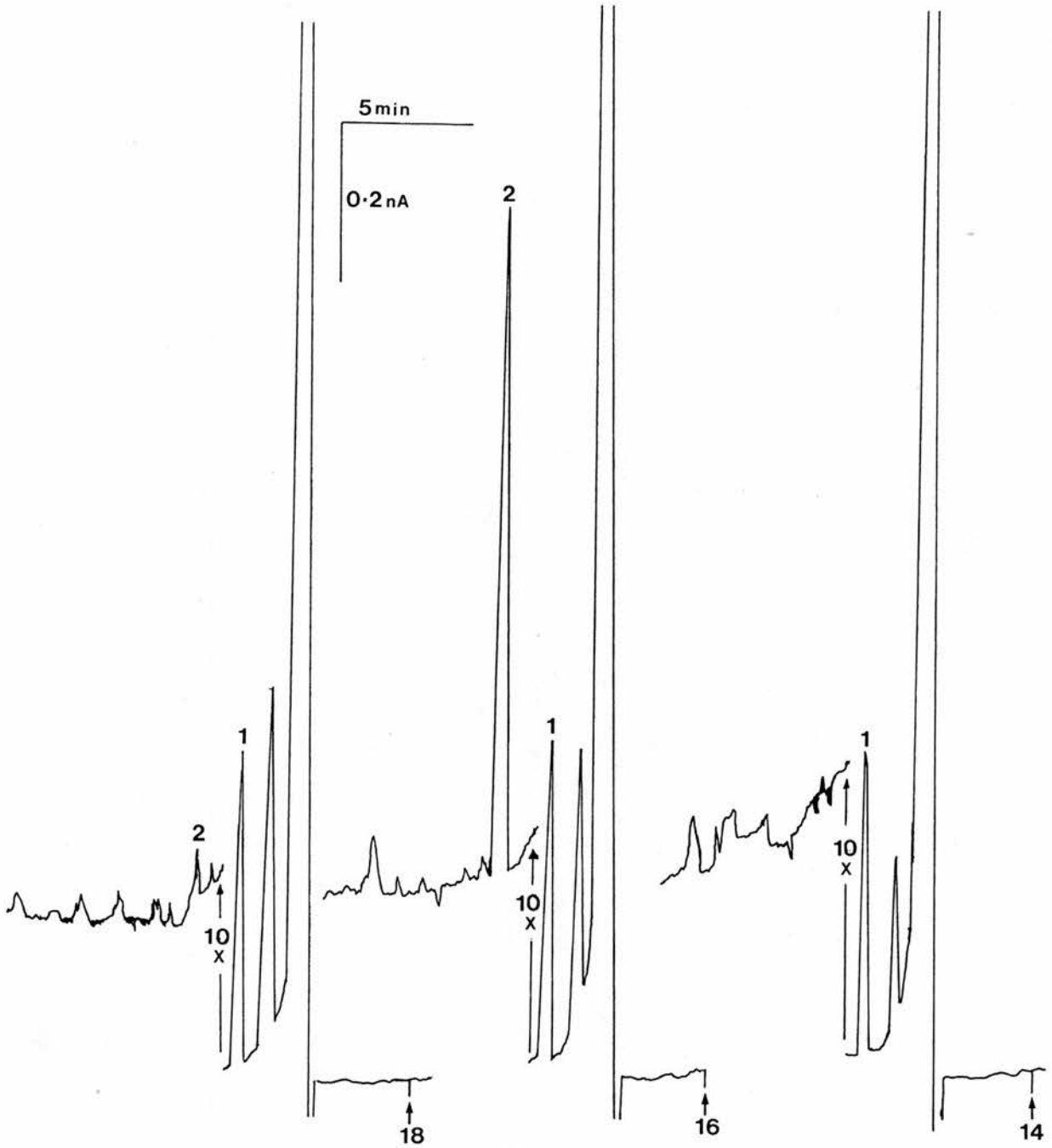


FIGURE 4.2: HPLC-ECD traces of median eminence (ME) superfusate samples. 4 ME tissue pieces superfused in Krebs solution (350µl/2 min) were stimulated twice (electrically). The traces shown above show the DA content of superfusate samples taken immediately before (14, numbers refer to superfusion time, min), during (16) and immediately after (18) the first electrical stimulation (S_1).

1 = Internal standard, dihydroxybenzylamine

2 = Dopamine.

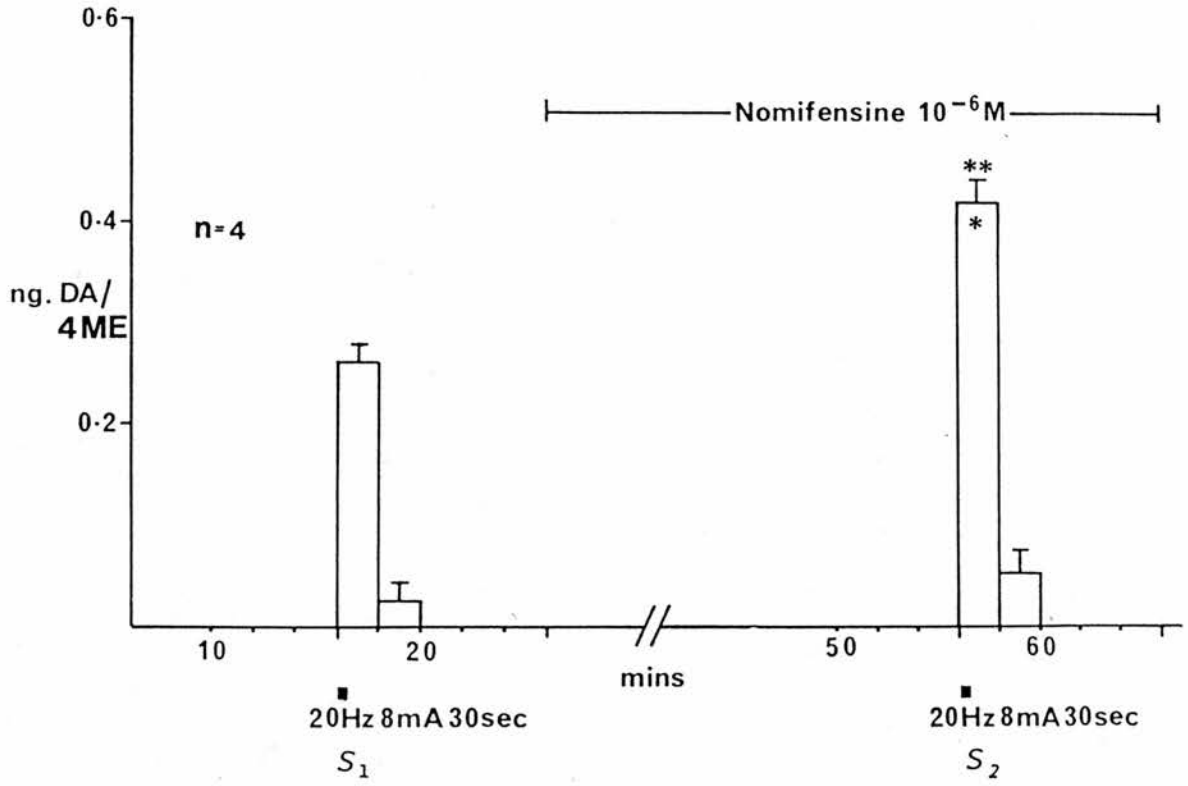


FIGURE 4.3: 4 median eminence tissue pieces superfused in Krebs solution were stimulated twice (electrically) at S_1 and S_2. After the control S_1, nomifensine (1μM) was added 30 min after the onset of superfusion. Nomifensine increased the overflow of DA at S_2 by about 50%.

(* $p < 0.05$, Student 't' test, one tailed vs. control S_2, see Figure 4.1; ** $p < 0.05$, paired Student 't' test, two-tailed vs. S_1)

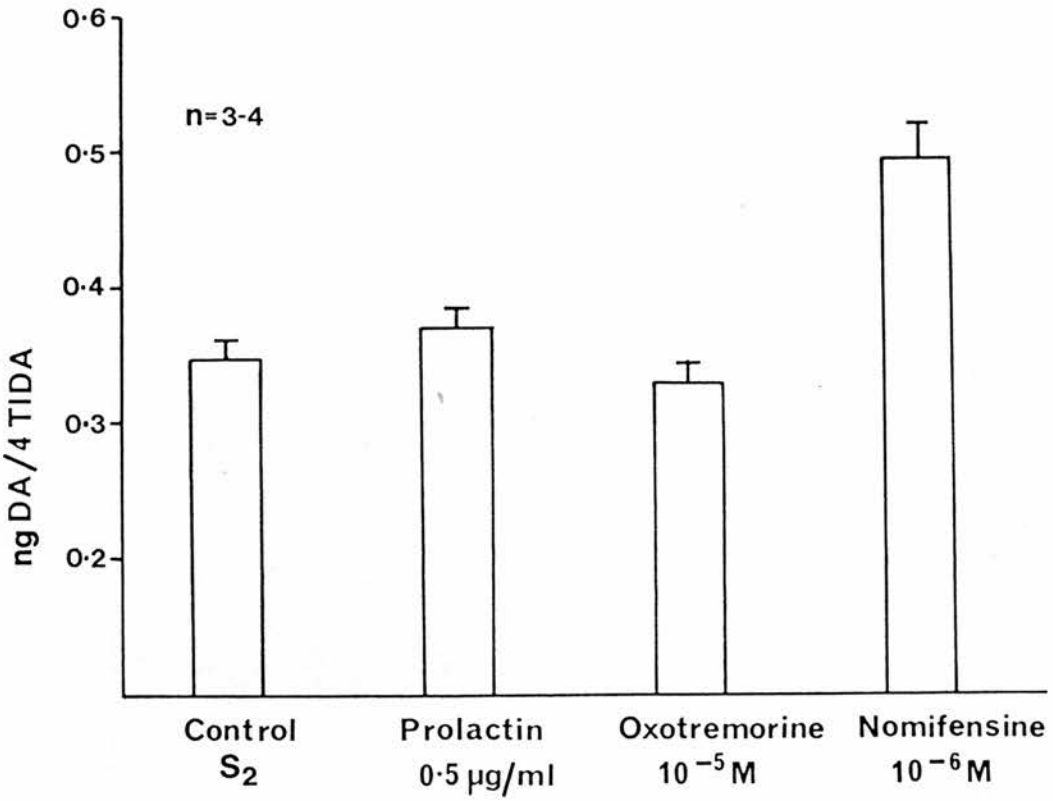


FIGURE 4.4: Summary of the electrically evoked overflow of DA from ME tissue superfused with Krebs solution.

Addition of prolactin (0.5µg/ml) or oxotremorine (10µM) 30 min after the onset of superfusion, failed to alter the evoked overflow of DA at S_2 (basal overflow remained below detectable levels).

As noted in Figure 4.3, nomifensine (1µM) increased the overflow of DA at S_2 by about 50%.

B) Discussion

The present experiments demonstrate an electrically evoked and K^+ evoked overflow of endogenous DA from the median eminence *in vitro*.

Recently the overflow of endogenous DA from the medial-basal hypothalamus (MBH) has been demonstrated by Sharman *et al.* (1982), using MBH tissue incubated in Krebs solution and the incubating fluid analysed by HPLC-ECD (see also Bennet *et al.*, 1981) and by Foreman and Porter (1981), using MBH tissue superfused with Krebs solution and superfusate analysed by a radioenzymatic assay. Despite differences in the dissection procedures, and in the experimental protocol (allowing for differences in the duration of stimulation), the values found by the above mentioned authors during high K^+ stimulation and those found during the present work, are roughly similar. This suggests that much of the DA in the MBH is localized around the ME.

As the K^+ and electrically induced overflow of DA did not differ markedly (c.f. striatal DA overflow, Figures 3.5 and 3.16), and nomifensine increased the overflow of DA by only 50%, the implication is that the ME lacks a high affinity DA uptake system, or that uptake plays a different role in transmitter metabolism.

Although a DA uptake system in the ME has been described (Cuello and Iversen, 1973), detailed studies by Demarest and Moore (1979) and Annunziato *et al.* (1980) have demonstrated that the ME DA uptake system has a very much lower affinity for DA than the striatal DA uptake system.

These observations are consistent with the postulated role of DA as a Prolactin Inhibitory Factor (McLeod, 1976).

Prolactin and oxotremorine failed to alter the evoked overflow of DA from the ME *in vitro*. Perkins and Westfall (1978, 1979) and Foreman and Porter (1981) have, however, recently reported facilitatory effects of these agents on the evoked overflow of DA *in vitro* from the MBH. Similarly, several authors (Gudelsky and Porter, 1980; Johnston *et al.*, 1980; Pilotte *et al.*, 1980) have demonstrated an increase of DA release and turnover *in vivo* with prolactin. It may be suggested, therefore, that the effects of prolactin and muscarinic agents are mediated by an action of these drugs on cell bodies rather than on nerve terminals (Yamada, 1975).

While the electrically evoked overflow of DA from the ME was similar to that induced from striatal slices in the presence of an uptake inhibitor (1μM nomifensine or LY5953A), the difference was that very little or no DOPAC overflow was detectable from the ME tissue. Umezu and Moore (1979) similarly found very low levels of DOPAC in the ME, which remained unaltered during treatment with haloperidol or reserpine, although the DOPAC levels in the striatum, olfactory tubercle, and hypothalamus were raised. Since Gudelsky and Moore (1977) showed an increased AMPT induced disappearance of DA from the ME with haloperidol, which implies increased DA utilization and synthesis, the implication is that the compartmentation and access of DA to MAO may differ in the ME from that seen in the striatum (Annunzaito, 1979).

4.2 The overflow of endogenous DA from cockroach salivary glands *in vitro*

A) Results

Cockroach salivary glands (CSG) superfused *in vitro* with oxygenated Ringers solution at room temperature, were stimulated twice

(electrically - 8-10mA at 20Hz for 30 sec). The basal overflow of DA was generally found to be at or below the limits of sensitivity of the HPLC-ECD (about 20 pg/sample) as was the 'metabolite peak'. The retention time of this metabolite peak corresponded to that of nADA (N-acetyl-dopamine) (see Figure 4.5).

During electrical stimulation there was a sharp rise in the overflow of DA (see Figure 4.6) accompanied by a slower increase in the overflow of nADA. A second electrical stimulation induced roughly the same increase in DA and nADA overflow as the first.

Omission of Ca^{++} ions from the Ringers solution during the second stimulation (S_2), abolished the electrically evoked overflow of DA and nADA.

An unidentified compound (hereafter referred to as R) was also detected in CSG extracts and in the superfusate of CSGs. R had a much longer retention time than NA, Adr, octopamine, DA or 5-HT, eluting in that order under the conditions employed. During electrical stimulation, R was also released, Ca^{++} dependently, with a slower time-course to that of DA (see Figure 4.6). The presence of large amounts of R in tissue extracts (although it was not possible to quantify R content of CSG's, R peak height was approximately an order of magnitude larger than that of DA) argued against it being a metabolite of a neurotransmitter (see Figure 4.7).

Since R may have been a secretory product of the salivary gland, attempts to induce R release by adding NA (10μM) to the superfusing Ringer solution proved unsuccessful. Similarly, blockade of post-synaptic receptors with phenoxybenzamine (10μM) failed to inhibit its release during nerve stimulation (the overflow of DA and nADA being unaltered as well).

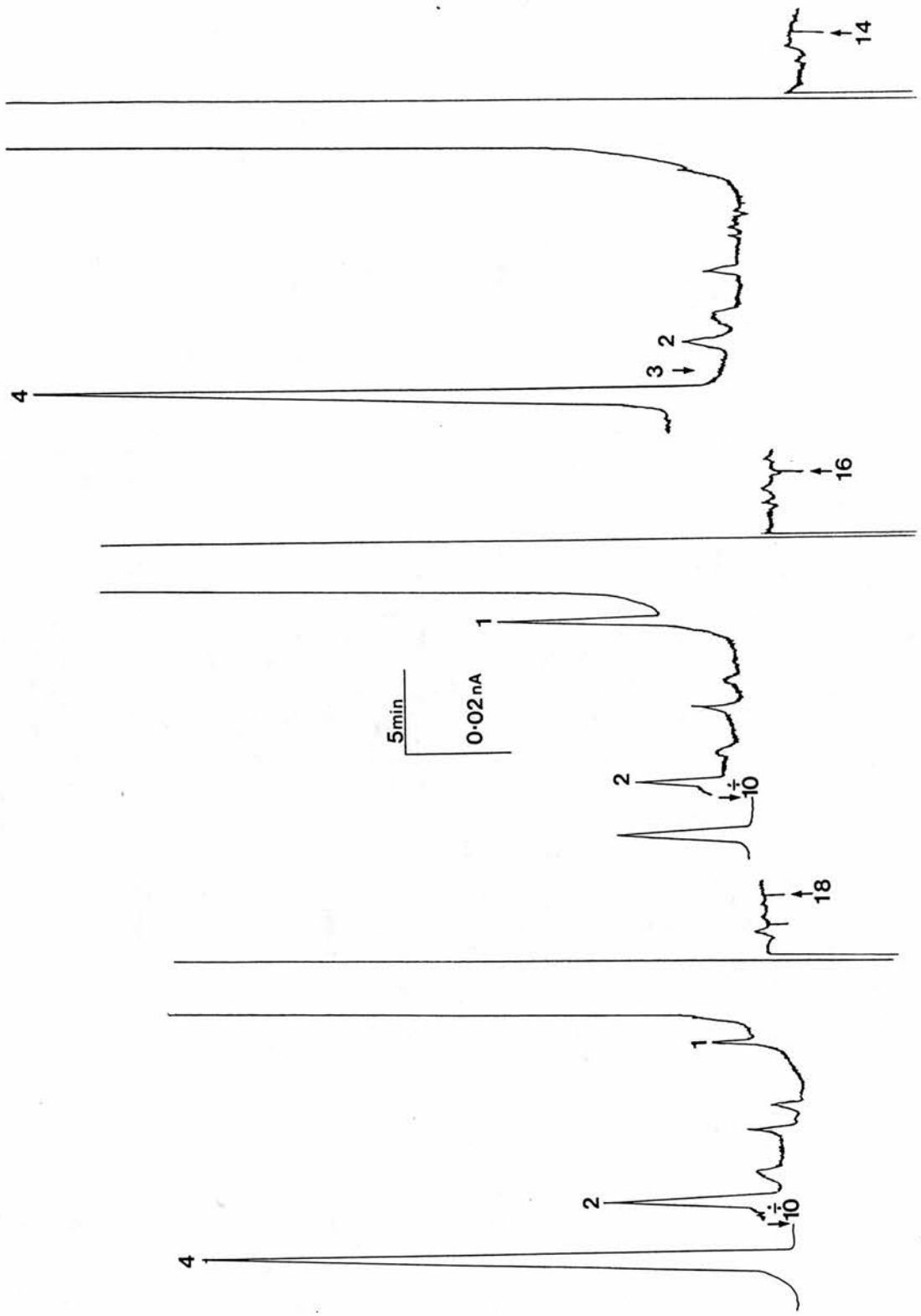


FIGURE 4.5: HPLC-ECD traces of cockroach salivary gland (CSG) superfusate samples (numbers refer to superfusion time). Samples taken before (14); during (16); and immediately after (18) electrical stimulation is shown.

1 = Dopamine
2 = N-acetyl dopamine
3 = retention time of 5-HT
4 = R

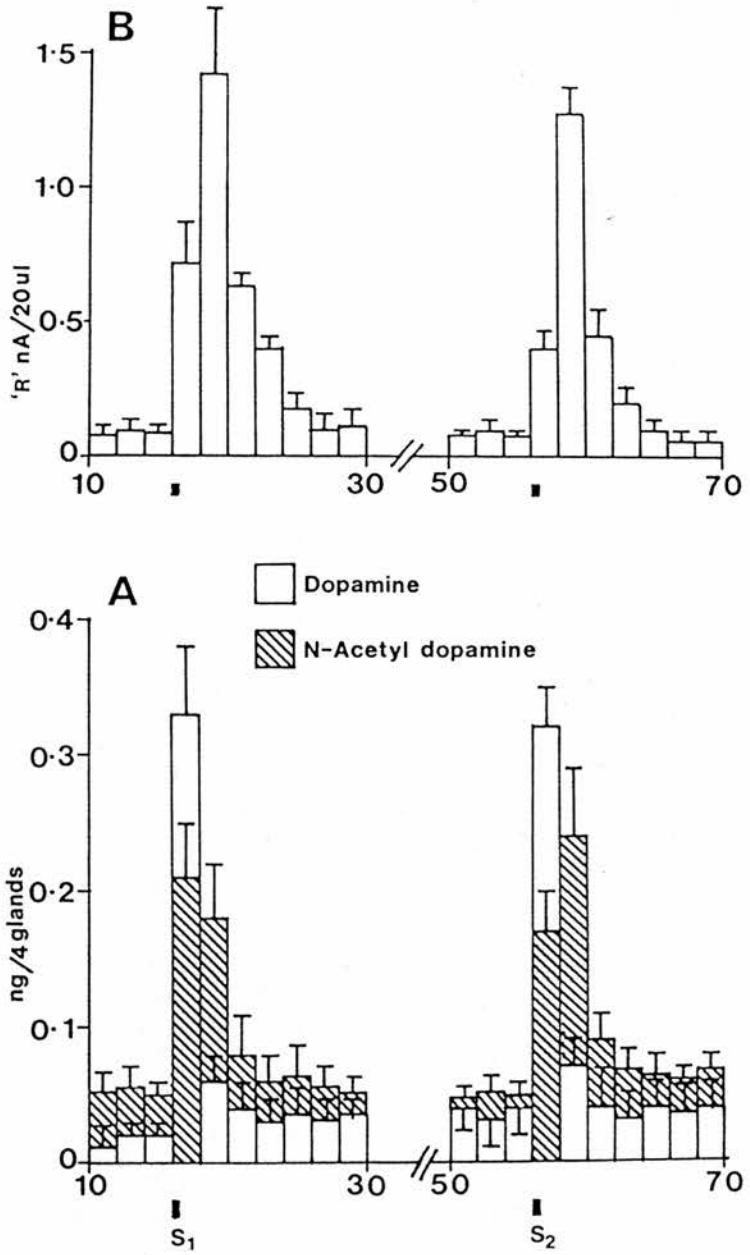


FIGURE 4.6: A. 4 cockroach salivary glands, superfused in oxygenated Ringers solution were stimulated twice (electrically) at 16 min (S_1) and 56 min (S_2). Superfusate samples collected at 2 min intervals were analysed for catecholamines by HPLC-ECD. Electrical stimulation induced a rapid rise (and subsequent decline of DA overflow as shown above. Omission of Ca^{++} from the Ringers solution prevent the increase in DA overflow due to electrical stimulation.

B. During the experiments described above, an unknown compound, R (see Figure 4.7), with a retention time greater than that of NA, Adr, DA or 5-HT was simultaneously 'released' during electrical stimulation. The evoked overflow of R was also found to be Ca^{++} dependent. The overflow of R, expressed as the limiting current obtained (nA) from a 20µl injection of the CSG superfusate into the HPLC-ECD, had a slower time course than DA overflow (cf. nADA in A).

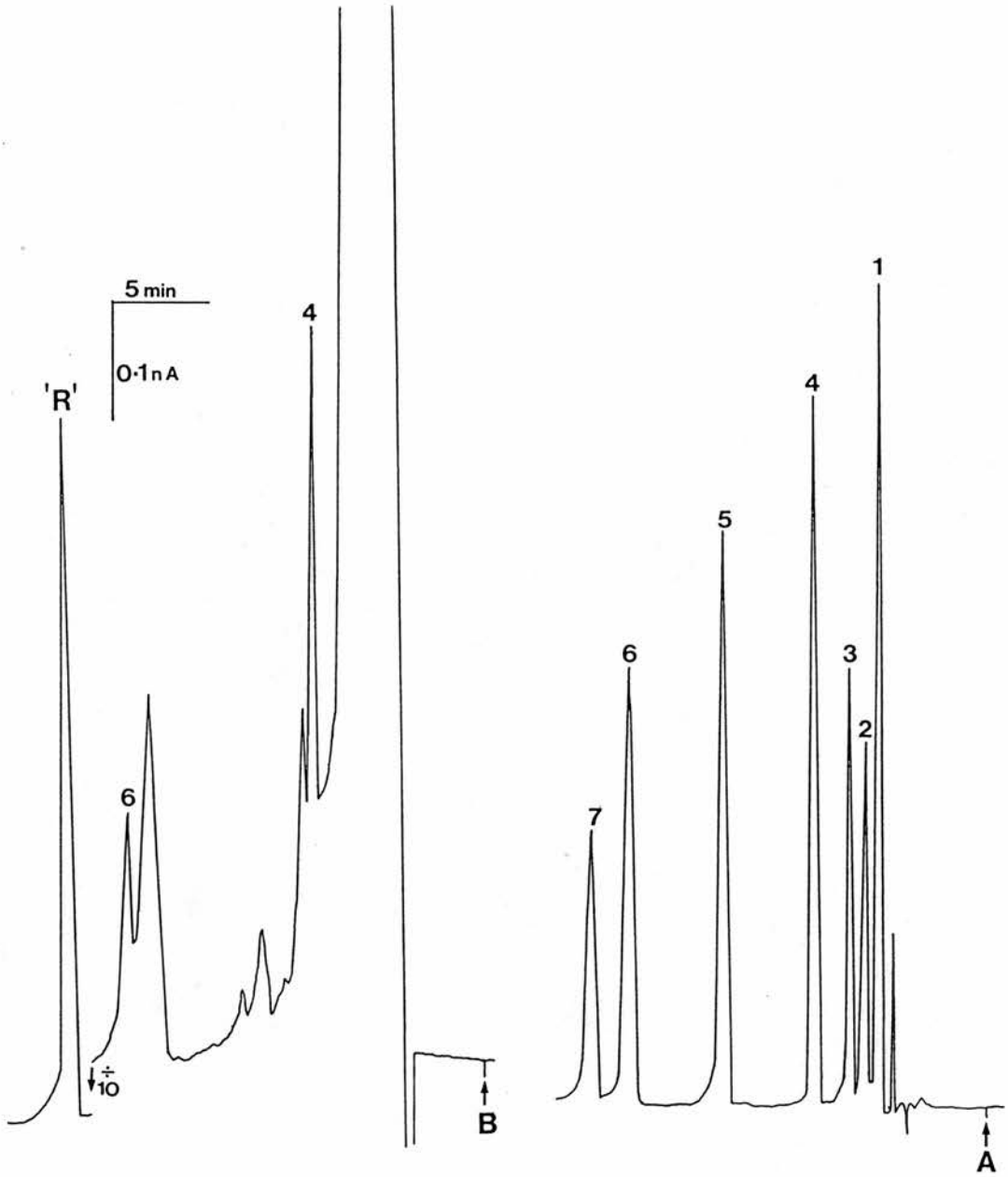


FIGURE 4.7: HPLC-ECD trace of the supernatent (20µl injection) of a cockroach salivary gland homogenate.

A. Standards: 1 = noradrenaline; 2 = adrenaline; 3 = dihydroxybenzylamine; 4 = dopamine; 5 = dihydroxyphenylacetic acid; 6 = N-acetyldopamine; 7 = 5-hydroxytryptamine.

B. 20µl of a CSG homogenate showing the position of R relative to the other catechol/indoleamines of interest.

Preliminary experiments with carbacol (10μM) did not show any alteration of the basal or evoked overflow of DA, nADA or R.

B) Discussion

The presence of catecholamines in the salivary glands of *Nauphoeta cinerea* Oliver has been demonstrated histochemically (Bland *et al.*, 1973); radioenzymatic measurement by Fry *et al.*,(1974) suggest that DA and not NA was the major catecholamine present. Recently, Mitchell and Williams (1981) reported the presence of DA and octopamine in the CSG by GCMS. The authors also reported a reduction of DA and octopamine content of the CSG after *in vitro* degeneration of nerve terminals with 6-OHDA. A neurotransmitter role for DA has also been suggested by electrophysiological methods, nerve stimulation and dopamine elicit similar changes in the membrane potential and conductance of acinar cells, which can be blocked by dopaminergic and α-adrenergic antagonists (Ginsborg *et al.*, 1976). Salivary secretion response of CSG *in vitro* can also be initiated by nerve stimulation or bath application of DA, NA, Adr and 5-HT, of which DA was found to be the most potent (House and Smith, 1978).

The pharmacology of the postsynaptic receptors on acinar cells indicates the presence of two types of receptors, a dopaminergic and a '5-HT-type' receptor (Bowser-Riley *et al.*, 1978; see House and Ginsborg, 1979 for a recent review). Light and electron micrscopy has revealed a dual innervation of each gland (see House, 1980).

The results of the present investigations support a neurotransmitter role for DA at the CSG neurosecretory synapse.

Large amounts of DA (1.9 ± 0.2 ng/gland) were shown to be present in the CSG, which could be released, Ca^{++} dependently, *in vitro*, by electrical stimulation. Since the ratio of released DA to

DA content of the preparation for the CSG is similar to that found for the ME preparation and much higher than that for the striatal tissue, it may be suggested that the CSG, like the ME, may only have a low affinity DA uptake system. nADA appeared to be the major metabolite of DA (Mir and Vaughn, 1981).

Preliminary attempts to isolate R by HPLC and characterize it by GCMS failed (probably due to its instability since it is easily oxidisable at +0.70), its identity remains unknown. Since R was present in what appeared to be relatively large amounts in tissue extracts and was released Ca^{++} dependently during electrical stimulation, a neurotransmitter for R is visualized.

4.3 Overflow of endogenous DA from the rat retina *in vitro*

A) Results

Rat retina, superfused with oxygenated Krebs solution at 37°C, were stimulated twice, at S_1 with light (flashing at 2-3Hz for 2 min) and electrically at S_2 (8-10mA, 20Hz, 30 sec).

Although the average DA content of each retina was relatively low (4.05 ± 0.55 ng/retina), the basal overflow of DA in the superfusate was relatively high around 0.07 ± 0.006 ng/retina/2 min. Although occasionally a peak eluting at the retention time of 3-MT was found, it was generally at or below the limits of sensitivity and so was not quantified (see Figure 4.8).

Light stimulation failed to modify the basal overflow of DA. During electrical stimulation, however, there was a marked increase in the overflow of DA, which declined slowly (see Figure 4.9), this electrically evoked overflow of DA was abolished when superfusing with Ca^{++} free Krebs.

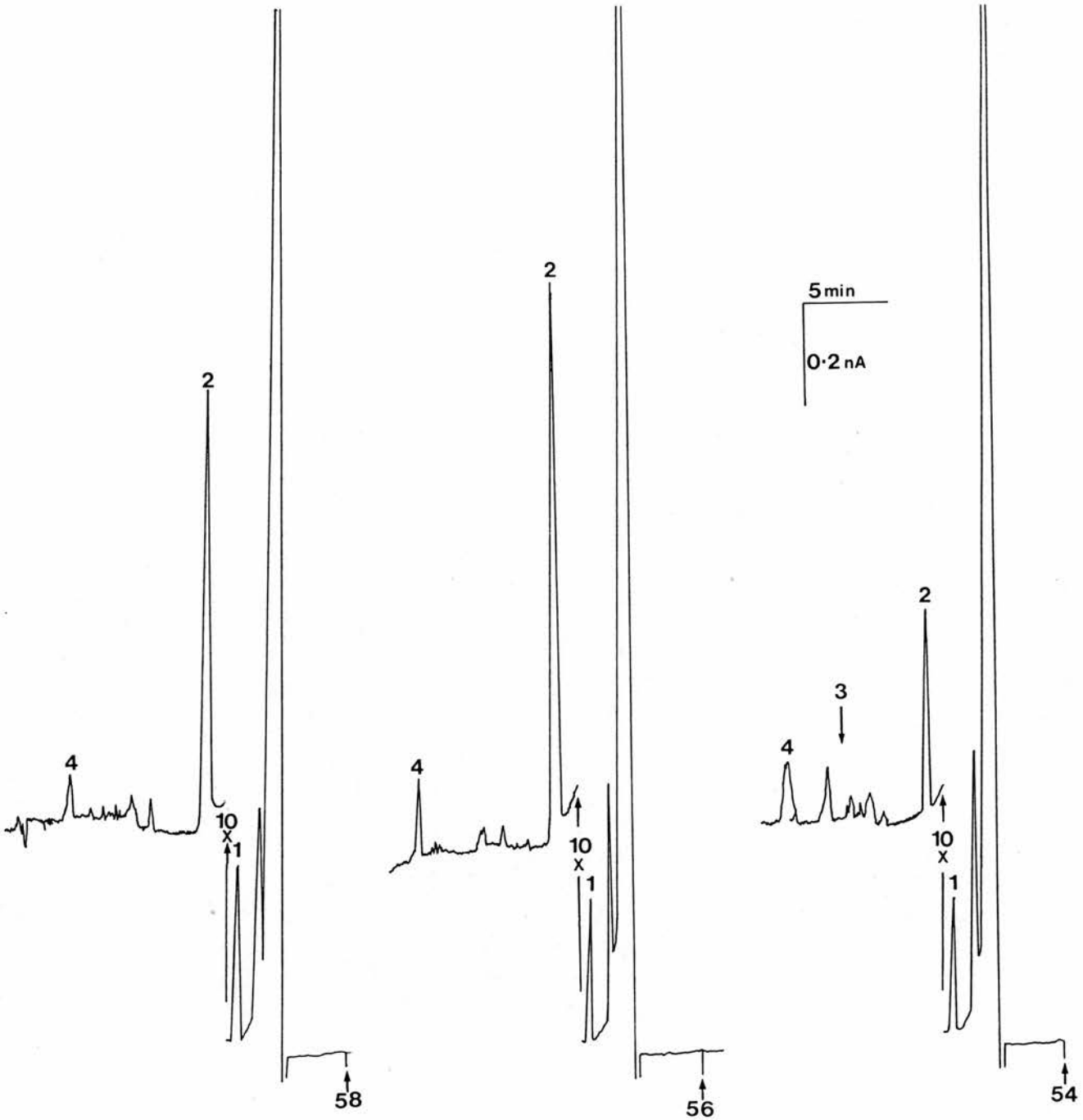


FIGURE 4.8: HPLC-ECD traces of the superfusate from retinal tissue (numbers refer to superfusion time of sample). Samples taken before (54), during (56) and immediately after (58) electrical stimulation (S_2) of retinal tissue (see Figure 4.9).

1 = Dihydroxybenzylamine, Internal standard
2 = Dopamine
3 = retention time of Dihydroxyphenylacetic acid
4 = 3-Methoxytyramine

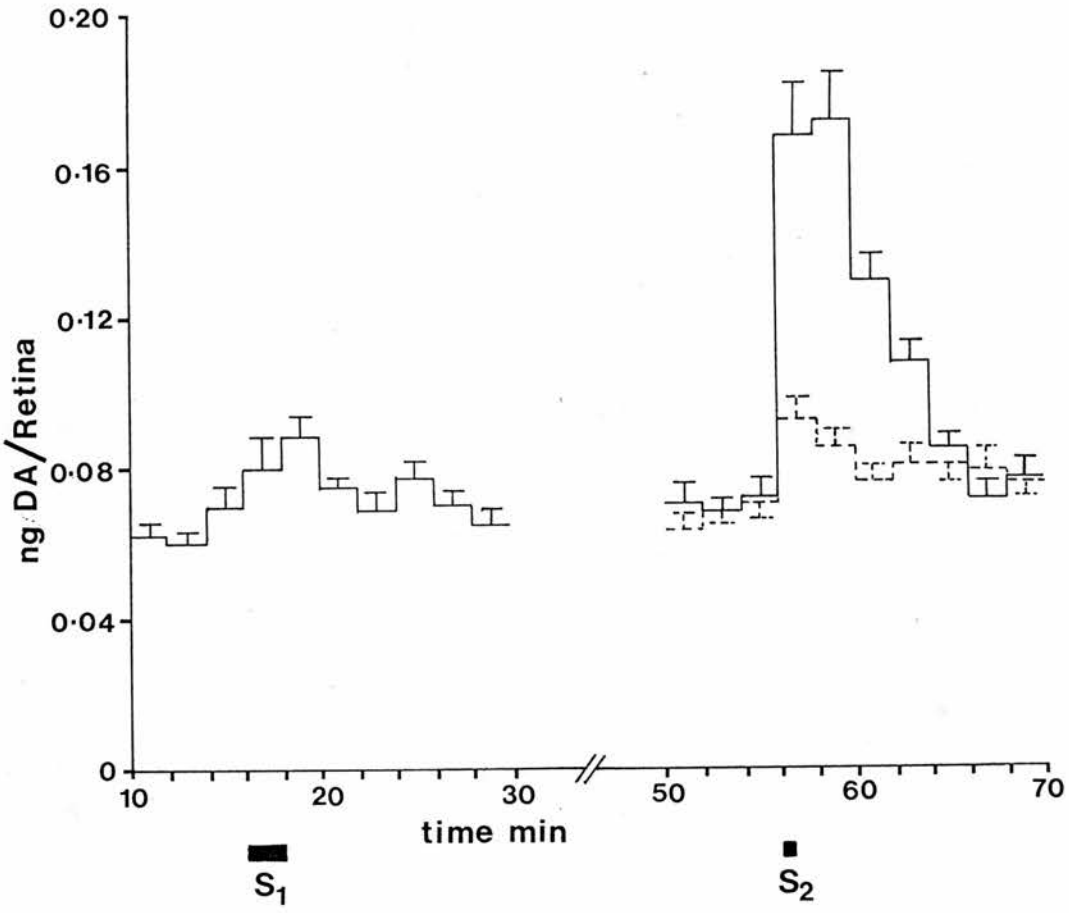


FIGURE 4.9: Retinal tissue superfused with Krebs solution was stimulated first with a light stimulus (strobe flashing at 2-3Hz for 2 min) at S_1 and then electrically at S_2 (20Hz, 8-10mA for 30 sec). The superfusate collected at 2 min intervals was analysed for its catecholamine content by HPLC-ECD.

The relatively high basal overflow of DA was unaffected by light stimulation at S_1. Electrical stimulation did evoke an increase in the overflow of DA, which was abolished when superfusing the tissue with Ca^{++} free (+0.1mM EGTA) Krebs solution (broken line histogram).

The DA content of the tissue at the end of the experiments was found to be 3.89 ± 0.54 ng/retina, and was not significantly different from the DA content of freshly dissected retina (4.05 ± 0.55 ng/retina).

The major metabolite of DA appeared to be 3-MT, although it was present in very small amounts. The DA content of the retina (3.89 ± 0.56 ng/retina) at the end of the experiments did not differ from that found in freshly dissected retina (see above).

B) Discussion

Dopamine in the retina of most mammalian species appears to be localized in the amacrine neurons within the inner nuclear layer (Dowling and Ehinger, 1978; Haggendal and Malmfors, 1965; Malmfors, 1963). A light induced release of ^{3}H-DA has been demonstrated (Kramer, 1971) associated with an increase in TH activity and DA utilization (Iuvone *et al.*, 1978; Morgan and Kamp, 1980).

However, I was unable to demonstrate a light induced overflow of DA from the rat retina *in vitro*, possibly because there was insufficient DA released due to inadequate stimulation or insufficient dark-adaptation of the preparation.

An electrically induced, Ca^{++} dependent, overflow of DA (with 3-MT as the major metabolite) was found, confirming the results of Dubocovich and Weiner (1981) on the overflow of ^{3}H-DA from the rabbit retina. The report by the above mentioned authors that the high basal overflow of ^{3}H was unaltered in the absence of Ca^{++} ions, was also confirmed during the present study. The DA content of the retina at the start and the end of the experiments did not differ, supporting the findings of Iuvone *et al.*,(1978) that the DA content of the dark adapted retina does not differ from that found in retina after light stimulation.

The present results confirm a neurotransmitter role for DA in the rat retina (for dopaminergic receptor characterization see Goodale *et al.*, 1980; Makman *et al.*, 1980) and it is suggested that differences

in the catabolism of DA between the retina and striatum indicate a difference in the subcellular compartmentation and metabolism of DA in the two tissues.

4.4 Estimation of dopamine in portal plasma

A) Results

Hypophysial portal blood collected as described in the methods (see page 62) was centrifuged and the plasma stored at -70°C until analysis. For analysis, after precipitation of the proteins, catecholamines were alumina extracted and analysed by HPLC-ECD.

To investigate the influence of hyperprolactinemia on DA release into portal blood, male rats were made hyperprolactinemic by implanting two pituitary glands under the kidney capsule a month prior to collection of portal blood. The DA/DOPAC content of portal plasma measured before, during and after stimulation of the ME (60Hz, 1mA, applied in trains of 30 sec over 30 min) were as follows:

Concentration of DA and DOPAC in hypophysial portal plasma (ng/ml, ± s.e.m.)

	Pre-stimulation	Stimulation	Post-stimulation
Control (n=6)			
DA	3.62 ± 0.35	2.66 ± 0.98	2.20 ± 0.07
DOPAC	7.20 ± 0.24	4.60 ± 0.28	7.00 ± 1.98
Hyper-Prl (n=5)			
DA	4.48 ± 0.34	4.56 ± 0.39	
DOPAC	9.47 ± 1.32	12.20 ± 1.10	

The recoveries found during these experiments tended to be rather low (20-60%). In an attempt to improve on the alumina extraction, and avoid batch variation, alumina was acid washed and dried several times. However, this did not lead to any significant improvement (see Figure 4.10).

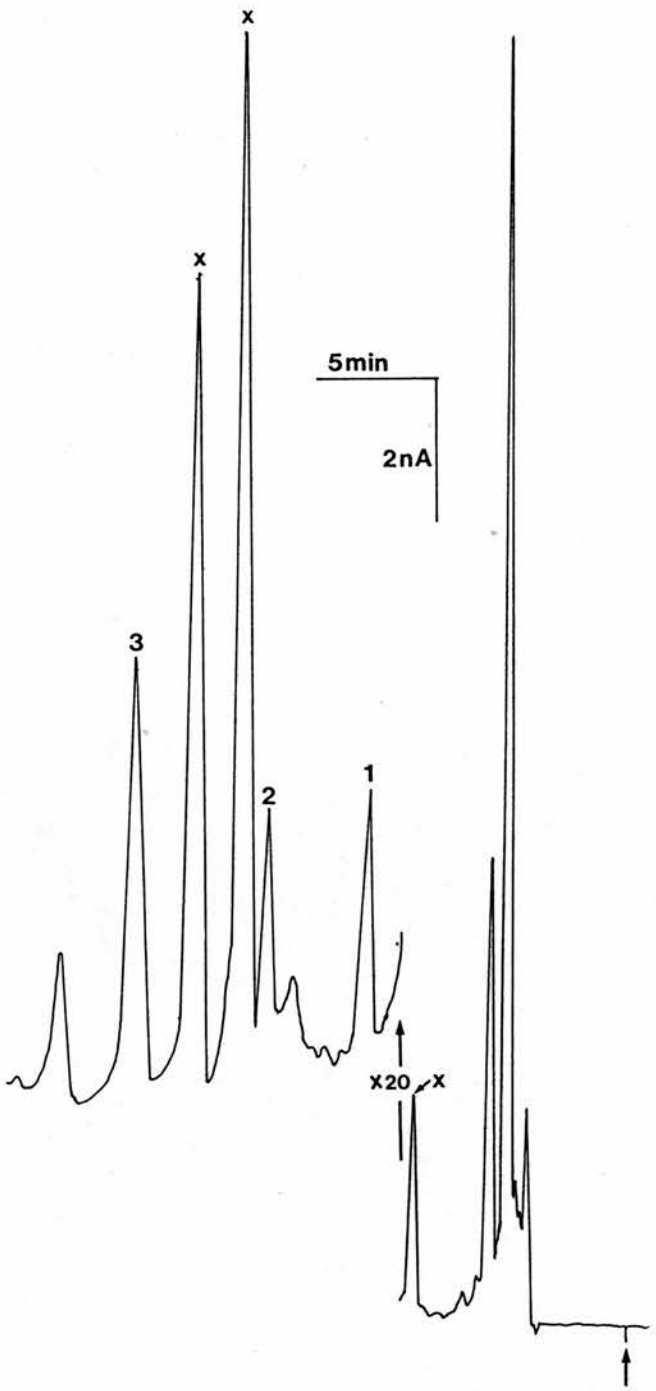


FIGURE 4.10: HPLC-ECD trace of an alumina extract of portal plasma.

1 = Dopamine
2 = Dihydroxyphenylacetic acid
3 = N-acetyl dopamine, Internal standard
X = Unknown peaks probably eluting off the alumina.

Preliminary experiments with cycling female rats suggested that the DA concentrations in portal plasma were higher at proestrus (a.m.) than at estrus or diestrus.

An attempt to look for differences in portal plasma levels of catecholamines after destruction of the ME dopaminergic nerve terminals by peripheral injections of 6-OHDA failed, due to inadequate recoveries from the alumina extraction, and the appearance of unidentified electroactive peaks eluting off the alumina. However, 48 hr after a peripheral injection of 6-OHDA (+ ascorbate and desmethylimapramine), the DA content of the ME tissue decreased from (control) 7.85 ± 0.63 ng/ME (n=8) to 4.72 ± 0.50 ng/ME (n=8).

B) Discussion

The limiting step of the HPLC-ECD assay for plasma catecholamines appears to be the alumina extraction step. Recoveries from different batches of alumina varied from 50-70% to 20-60%; a large solvent front (perhaps due to uric acid and its metabolites, C.A. Marsden, personal communication) - and the appearance of unknown peaks (see Figure 4.10) eluting off the alumina (Falconer *et al.*, 1982) severely limited the sensitivity of the assay.

Studies on hyperprolactinemic male rats suggest that despite a lowered ME DA content (Simpkin *et al.*, 1982), DA release into portal plasma is higher than control even during stimulation of the ME. The relevance of the stimulation induced decrease of DA release in control animals remains obscure.

Peripheral injections of 6-OHDA (Smith *et al.*, 1982) lowered the DA content of the ME by about 50%, despite the possibility that part of the DA measured from the 6-OHDA lesioned ME may have been the DA accumulation in the lesioned end of the ME axons (C.G. Smith, personal communication).

The findings of Smith *et al.*, (1982) that prolactin release remains unaltered after destruction of the ME dopaminergic nerve terminals, despite a decrease in the ME DA levels (present thesis) and reduction in ME fluorescence (Smith *et al.*, 1982), raises the possibility that DA accumulated in the 'cut' DA axons is releasable as suggested by the peripheral experiments of Esquerro *et al.*,(1980a,b).

CHAPTER V

Summary and Conclusions

From the evidence presented in this thesis, the following sequence for the metabolism of DA in the striatum is suggested:

DA is synthesized (from tyrosine by tyrosine hydroxylase and DOPA decarboxylase) presumably at a site at which it has access to MAO. This site may be the 'newly-synthesized releasable pool' of DA, or the 'cytoplasmic pool' as suggested by Broxterman *et al*. (1979) (see Figure 5.1).

The rather large amount of basal DOPAC overflow found during the present thesis, implies that a significant proportion of the newly synthesized DA is immediately deaminated. The remaining newly-synthesized DA is then available for release or transfer to the storage pool. As time goes on, the possibility that a newly-synthesized (or recently take up) molecule of DA is released, declines; while the possibility that it is deaminated or stored increases. The release and uptake of DA, therefore, appears to involve mainly the newly-synthesized releasable pool of DA, which only slowly equilibrates with the larger storage pool.

Most of the released DA (>90%) is taken back up by the nerve terminal, while much of it is recycled, presumably initially entering the releasable pool, some of it may be deaminated.

However, several major points about the compartmentation of DA remain to be determined; especially the size of the releasable pool, which is most probably less than 40% but more than about 5% of the total tissue DA.

DOPAC was found to be the most abundant metabolite of DA in the striatum, however changes in the rate of formation of DOPAC appear to relate better to changes in the rate of synthesis and not the release of DA. It has been further suggested that HVA is formed

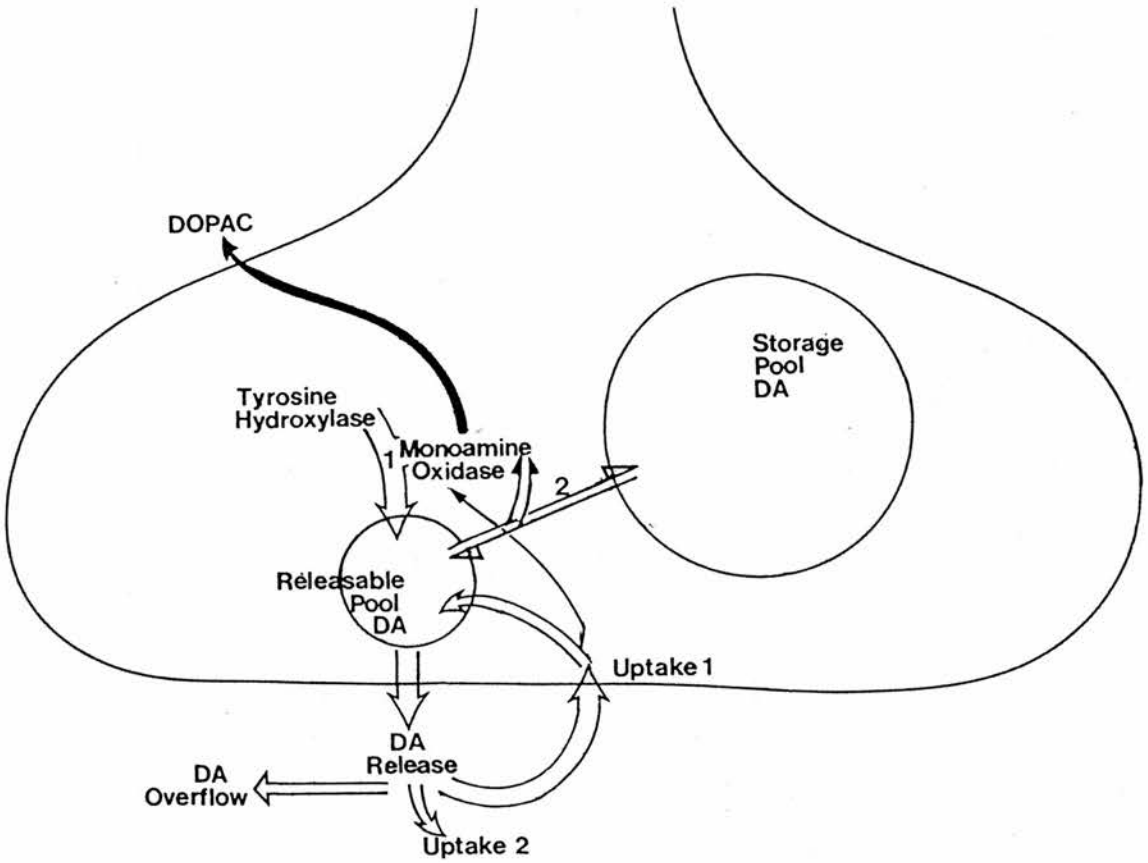


FIGURE 5.1: Schematic representation of the intraneuronal flux of dopamine in striatal nerve endings.

Newly synthesized dopamine initially enters the releasable pool. A large part of this newly-synthesized dopamine appears to be deaminated either at '1 - the cytoplasmic pool', or because DA within the releasable pool has access to MAO. The releasable pool of DA (about 5-30% of total tissue DA) is then available for release. Release appears to take place preferentially from this pool. DA taken up at uptake 1 (neuronal uptake) also appears to initially enter the releasable pool, little of it being deaminated.

The rate of transfer of DA between the releasable and storage pools (2) appears to be very slow, and inadequate to supply sufficient DA for release in the absence of DA synthesis. This may be an important point for the regulation of DA storage by presynaptic receptor mechanisms.

Presynaptic receptors also appear to regulate the rate of synthesis of DA, and the release of newly-synthesized DA.

mostly extra-neuronally and is derived mainly from DOPAC. An extra-neuronal site of 3-MT formation, mainly from released DA, has also been suggested.

The release of DA appears to increase with increasing frequency of stimulation, with a maximum around 20Hz. A depression of release per impulse with increasing train length was demonstrated, although the effect of presynaptic receptor mediated mechanisms on this depression of release per impulse remains to be investigated.

There is now increasing evidence in the literature that dopaminergic presynaptic receptors can depress the release of (preincubated) ^{3}H-DA, and increase the 'turnover' of DA (see Table 1, page 31, for references). This implies that the presynaptic effects of dopaminergic agents on the release of DA are to some extent independent of de novo synthesis. However, a further implication is that the release of newly-synthesized DA should also be increased, since it has been suggested that newly-synthesized DA is preferentially released. Evidence for this was obtained during the present thesis, and it was found that the effects of dopaminergic agents on endogenous DA release were more marked than the effects on ^{3}H release. The above mentioned findings suggest that ^{3}H overflow, after preincubation of the tissues with ^{3}H-DA, is not a reliable index of total endogenous DA overflow.

Similarly, muscarinic agonists increased the release of endogenous DA to a much greater extent than the release of ^{3}H. A similar small facilitation of ^{3}H release from striatal synaptosomes has recently been reported (Marchi *et al.*, 1982). This further emphasises the view that the major mechanisms affected by presynaptic receptor mediated effects are the rate of synthesis and the release of newly-synthesized DA, and perhaps to a lesser extent the transfer of DA between the releasable and storage pools.

The control of release and metabolism of DA from the other DA rich areas examined appeared to differ from that found in the striatum in several respects:

1. The median eminence nerve terminals appear to lack a high affinity DA uptake system, and the intraneuronal DA appears to have a more limited access to MAO. These nerve terminals did not appear to have active muscarinic or prolactin presynaptic receptors, and so the effects of these drugs on the release of DA are probably mediated via an action on the cell bodies of the tuberoinfundibular neurons.

2. The retinal intraneuronal stores of DA also appear to have only limited access to MAO, the major metabolite of released DA is most likely to be 3-MT. The retinal dopaminergic neurons appear to have a much higher basal overflow of DA than striatal or ME nerve terminals, the implications of which are as yet obscure.

3. Further evidence for DA being a neurotransmitter in the cockroach salivary gland was obtained as endogenous DA could be Ca^{++} dependently released by electrical stimulation of the glands *in vitro*. The major metabolite of DA in this tissue appears to be nADA. The existence of another unidentified transmitter, R, was suggested.

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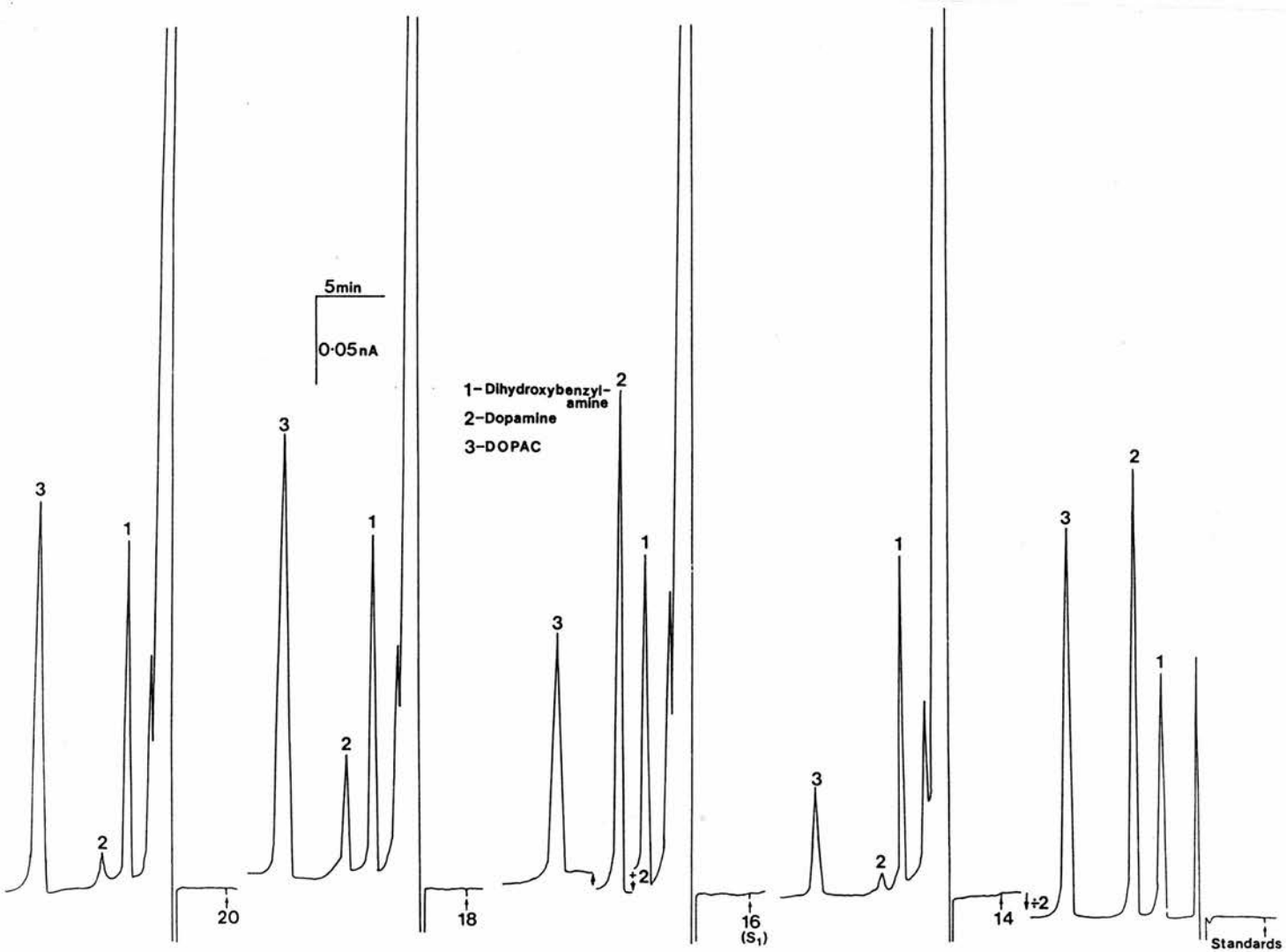
APPENDIX I:

Calculation of Results


5min
0·05nA
1–Dihydroxybenzyl-amine
2–Dopamine
3–DOPAC
3
2
1
20
18
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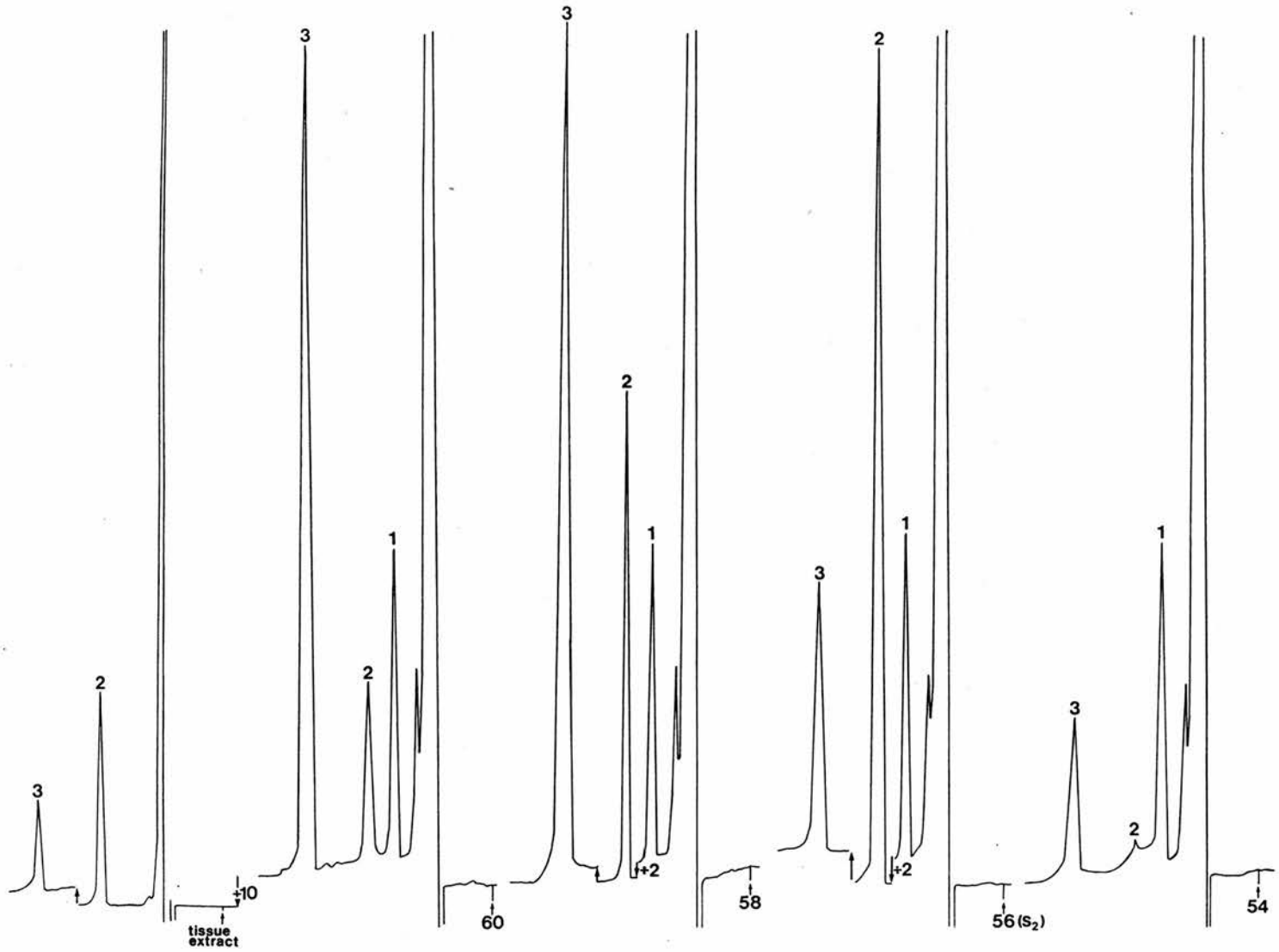
(S1)
14
Standards
÷2


3
2
3
2
1
3
2
1
3
2
1
3
2
1
3
2
1
+10
tissue extract
60
+2
58
+2
56 (S2)
54

Examples of HPLC-ECD traces of superfusate samples (numbers refer to superfusion time, stimulations were carried out at 16 and 56 min) from one experiment are shown on the preceeding two pages.

All results were calculated using the following equation:

$$\text{ng/mg protein/sample} = \frac{\text{peak height of sample}}{\text{peak height of standards}} \times \text{amount of standards} \times \frac{\text{average flow rate/2 min}}{\text{injection volume}} \times \frac{1}{\text{proteins}}$$

Only experiments with more than a 90% recovery of the internal standard were used, corrections for recovery were not made.

An example of the format in which the results were stored is shown overleaf (calculated from the experimental results shown on the previous two pages).

Date: 21-3-82 Chamber = A2 Gain of HPLC-ECD = 0·5nA/V

Paper speed = 12cm hr^{-1}

Flow rate of superfusing Krebs = 650µl/2min.

Protein content of tissue = 1·60mg

Drugs used = NEOSTIGMINE, 1µM. Time of addition of drugs = 30min.

Standards (peak height, amount injected)

Internal standard, (DHBA) = 0.54 , 0.2 ng

DA = 1.02 , 0.2 ng

DOPAC = 0.86 , 0.2 ng

Stimulation parameters = 25mM K$^+$, 2min Injection volume = 20µl.

Superfusion time	ng/mg protein/sample DA	DOPAC
10	0.02	0.55
12	0.04	0.56
14	0.06	0.59
16	4.77	1.25
18	0.53	2.41
20	0.08	2.08
22	0.08	1.86
24	0.09	1.57
26	0.07	0.99
28	0.06	0.91
30	0.06	0.89

Superfusion time	ng/mg protein/sample DA	DOPAC
50	0.04	0.85
52	0.06	0.89
54	0.07	0.85
56	7.80	1.42
58	4.47	4.63
60	0.85	4.54
62	0.19	4.02
64	0.09	3.71
66	0.08	2.90
68	0.08	2.10
70	0.09	1.54

Average basal overflow before (ng/mg protein/2 min)

S_1 = DA: DOPAC: 0.57

S_2 = DA: DOPAC: 0.86

Evoked overflow (ng/mg protein)

S_1 = DA: DOPAC: 3.97

S_2 = DA: DOPAC: 8.04

Tissue content at the end of the experiment (ng/mg protein)

DA = 125.6 DOPAC = 6.54

APPENDIX II:

Published Papers

Regional changes in cerebral glucose utilization in kindled rats during convulsions

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Rats were kindled (Goddard, McIntyre & Leach, 1969) from the left amygdala by a daily one second pulse at 60 Hz with a current intensity of 300 μa (Farjo & Blackwood, 1978).

To identify the brain regions in which glucose utilization and hence functional activity is altered in kindling, a modification of the technique developed by Sokoloff *et al.* (1977) was used. Two minutes after the tail vein injection of 2-deoxy-D-[1-^{3}H]-glucose (200 μCi/Kg) one group of kindled rats received a stimulus which provoked a generalised convulsion. A second group of kindled rats and groups of sham operated and unoperated controls received no stimulus. All animals were killed and decapitated at 45 min after injection, the brains rapidly removed and frozen at −20 C. One mm coronal slices were prepared and specific regions removed with the aid of a dissecting microscope. The degree of labelling (counts min^{-1} mg protein^{-1}) for each brain region was expressed as a percentage of the value for the visual cortex in each animal.

In the brain areas shown in Table 1, glucose utilization was significantly increased in the kindled rats which had convulsed, when compared with the other three groups. Kindled rats which had not been convulsed did not differ significantly from the sham operated or unoperated groups. No significant difference betweed the groups was found in fronto-parietal cortex, caudate/putamen, thalamus, cerebellar hemispheres, dentate nucleus, brain stem reticular formation, inferior colliculus or olfactory bulb.

These results suggest that generalised seizures, associated with kindling of the amygdala selectively involves sub-cortical structures listed in Table 1.

Table 1

	Kindled (convulsed) $n = 9$ $\bar{x} \pm s.d.$	*Kindled (not convulsed)* $n = 6$ $\bar{x} \pm s.d.$	*Sham operated* $n = 9$ $\bar{x} \pm s.d.$	*Unoperated* $n = 11$ $\bar{x} \pm s.d.$	*Analysis of Variance F ratio*
R. Amygdala	82 ± 5.1	74 ± 5.5	71 ± 5.3	69 ± 4.7	6.9a
L. Amygdala	83 ± 5.1	72 ± 5.5	72 ± 4.6	70 ± 5.6	9.4a
R. Hippocampus	92 ± 11	79 ± 5.1	75 ± 3.9	70 ± 3.1	10.8a
L. Hippocampus	93 ± 7.1	79 ± 3.4	75 ± 3.5	71 ± 4.4	14.4a
R. Hypothalamus	83 ± 5.6	77 ± 3.6	73 ± 5	66 ± 6.6	11.8a
L. Hypothalamus	83 ± 5.5	76 ± 2.6	72 ± 4.9	67 ± 7.2	10.5a
R. Septal Region	85 ± 5.3	77 ± 4.7	72 ± 5.5	65 ± 6.7	11.9a
L. Septal Region	91 ± 9.1	76 ± 4.2	74 ± 7.6	66 ± 5.7	13a
R. Subst. Nigra	87 ± 9.9	77 ± 5.4	64 ± 10.4	66 ± 5.2	9.1a
L. Subst. Nigra	89 ± 6.2	72 ± 4.2	66 ± 12.8	65 ± 5.3	9.8a
R. Superior Colliculus	108 ± 6.9	94 ± 6.3	93 ± 8.1	88 ± 8.9	9.2a
L. Superior Colliculus	106 ± 6.6	91 ± 6.7	91 ± 5.5	89 ± 5.5	7.5b

The accumulation of tritium in each brain region was measured as (ct/min)/mg protein and then expressed as a percentage of the value for the visual cortex in each rat. The table gives the mean and standard deviation of this percentage. The analysis of variance showed a significant difference between groups in all these areas. a. $P < 0.0005$, b. $P < 0.001$.

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Regional changes in cerebral glucose utilization associated with amygdaloid kindling and electroshock in the rat*

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(Accepted July 23rd, 1981)

Key words: amygdala — 2-deoxyglucose — electroconvulsive shock — epilepsy — hippocampus — kindling

A method using [^{3}H]2-deoxyglucose was used to identify brain areas activated during partial and generalized amygdaloid kindled seizures and generalized seizures following electro-convulsive shock in rats. The amygdala, hippocampus, septal nuclei and hypothalamus were bilaterally activated in kindled convulsions. Electroshock caused a more widespread involvement including the thalamus, striatum, reticular formation and cerebellum. Changes in the amygdala and hippocampus but not hypothalamus, were found after partial kindling.

Methods which employ [^{14}C]2-deoxyglucose as described by Sokoloff[12] have been used to study the functional anatomy of seizures in a number of experimental models of epilepsy including kindling[1–3,6,7,9]. In this study a relative measure of glucose utilization in selected brain regions has been used to investigate patterns of activity following seizures induced by amygdaloid kindling, partial kindling and electroshock in rats. Kindling described by Goddard[5] is a lasting change in brain function following repeated spaced low intensity electrical stimulation.

Male Wistar rats (180–200 g) were prospectively allotted to 6 groups as follows. Group 1: 11 unoperated controls. Group 2: 9 operated controls which were implanted with an electrode in the left amygdala but received no stimulation. Group 3: 6 fully kindled animals did not receive a stimulus and did not convulse following deoxyglucose injection. Group 4: 6 animals kindled partially to stage two[10] (after 4–8 stimulations) had partial seizures with repetitive jaw movements following deoxyglucose injection. Group 5: 9 animals were fully kindled from the left amygdala (mean 16.1, range 12–23 stimulations). Each had a generalized seizure following deoxyglucose injection. Group 6: 10 unoperated animals received electroconvulsive shock following deoxyglucose injection (150 V sine wave for 1 s; maximum 50 mC).

The implantation of bipolar stainless steel electrodes and the kindling procedure were as previously described[4]. The kindling stimulus was a 1 s train of monophasic

* A preliminary report of this research was made at the British Pharmacological Society meeting in September 1979.

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TABLE I

Relative glucose utilization in various brain regions expressed as a percentage of the value obtained in each animal for the occipital cortex

Figures in each column are: mean, S.D., n.

	Unoperated			*Sham operated*			*Kindled (no convulsion)*			*Partially kindled*			*Kindled (convulsed)*			*E.C.S.*		
Parietal cortex R	100.1	5.1	11	102.7	8.9	9	101.8	6.0	6	96.2	7.4	6	98.6	3.7	9	101.5	4.0	8
Parietal cortex L	96.3	6.4	11	100.2	13.0	9	92.5	9.1	6	93.8	4.5	6	103.2	9.9	9	100.6	4.3	10
Amygdala R	69.1	4.9	11	70.9	5.6	9	73.8	6.0	6	80.2	6.0	6	82.2	5.4	9	93.3	7.7	10
Amygdala L	70.3	6.0	10	72.4	4.9	9	75.3	6.0	6	80.7	6.8	6	83.4	5.5	9	94.0	9.6	10
Hippocampus R	70.0	3.2	11	74.7	4.1	9	78.8	5.6	6	84.0	13.2	6	91.6	11.7	9	102.7	9.3	10
Hippocampus L	71.3	5.8	10	75.1	3.7	9	81.0	7.6	6	83.0	10.4	6	92.6	7.5	9	101.6	8.3	10
Hypothalamus R	66.0	6.9	11	72.8	5.3	9	76.5	3.9	6	79.0	3.2	6	82.3	6.1	9	86.0	8.5	10
Hypothalamus L	66.6	7.6	11	72.3	5.2	9	76.3	2.9	6	79.2	8.4	6	81.7	6.1	9	86.4	9.7	10
Septal nyclei R	65.4	7.0	11	72.4	5.9	7	76.5	5.1	6	—			85.1	5.7	7	90.0	7.4	10
Septal nuclei L	66.2	6.0	11	74.4	8.2	7	76.3	4.6	6	—			90.9	9.8	7	91.4	7.0	10
Thalamus R	85.5	8.0	11	91.1	5.1	9	96.0	9.6	5	—			93.0	8.4	8	95.0	7.6	10
Thalamus L	81.5	6.4	11	89.0	5.3	9	94.8	7.4	6	—			94.9	7.2	9	94.5	9.0	10
Reticular R	77.0	6.8	11	78.7	8.3	7	90.0	3.4	6	—			87.1	10.7	9	94.9	8.8	9
Formation L	80.9	9.4	11	82.0	7.6	7	87.2	4.4	5	—			88.0	8.0	9	93.1	10.7	9
Cerebellar R	68.1	5.2	11	73.4	8.6	9	74.4	6.4	5	—			78.4	7.8	9	81.5	11.0	10
Hemispheres L	69.2	8.8	10	72.3	7.3	7	77.8	8.8	5	—			80.6	7.7	9	84.0	16.1	10
Striatum R	83.1	6.9	11	85.9	6.2	9	94.6	7.5	5	—			94.2	6.1	9	95.6	9.8	10
Striatum L	84.7	5.0	11	88.3	7.7	9	89.8	9.0	6	—			91.8	5.2	9	98.9	7.9	10

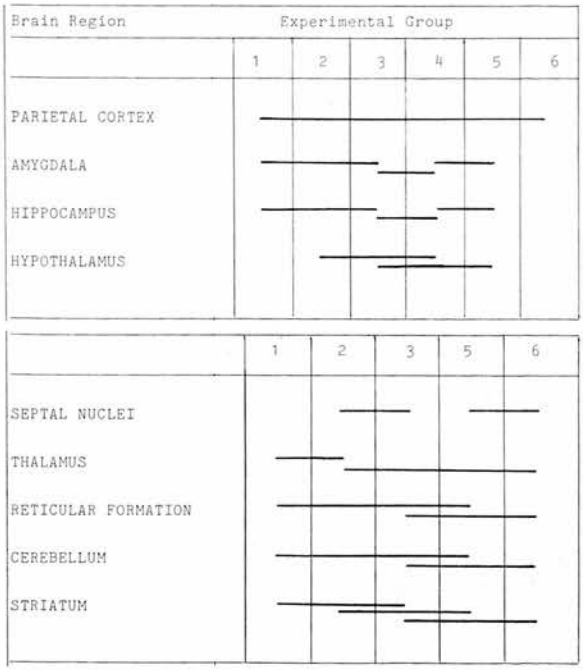

Fig. 1. A diagrammatic representation of the results of a multiple comparison test (Student–Newman–Keul) applied to the mean relative glucose utilization in the brain regions given in Table I. Groups: 1, unoperated controls; 2, operated controls; 3, kindled rats not convulsed after deoxyglucose injection; 4, partially kindled; 5, kindled. Convulsed after deoxyglucose injection; 6, electroconvulsive shock. The mean glucose utilization in any two groups enclosed by the range of any one line are not significantly different. Groups not enclosed by the same line have mean glucose utilizations which differ significantly at the 5% level.

pulse at 60 Hz and 300 μA. At least one week elapsed between the completion of kindling (3 consecutive seizures) and the deoxyglucose injection. Aliquots of 200 μCi/kg D-[1-^{3}H]2-deoxyglucose (Radiochemicals Amersham) in 300 μl saline were injected by tail vein and after 2 min animals in groups 4 and 5 received a kindling stimulus and group 6 an electroshock. In group 4 mean afterdischarge duration was 46 s (range 24–84 s) and in group 5 afterdischarge duration was 78 s (range 25–120 s). In group 6 the generalized seizure lasted 20–90 s (mean 64 s).

Following ictus animals were unrestrained and individually housed for 45 min, then killed by a blow on the thorax, decapitated and the brain quickly removed and frozen to —74 °C (Drikold) before being hand cut into approximately 1 mm slices. The selected brain regions shown in Table I were dissected at 0–4 °C under a dissecting microscope. Individual slices were thawed for only 2–4 min. The septal nuclei were all those structures enclosed by the lateral ventricles, corpus callosum and anterior commisure. The striatum included the globus pallidus.

Brain samples were homogenized in 100 μl distilled water in Ependorf microtubules and after removal of a sample for protein estimation[8] the homogenate was added to 10 ml Scintillator (NE 260 New England Nuclear) for liquid scintillation

counting. Activity in each brain region was expressed as cpm per mg protein. Because during seizure a steady state for glucose utilization does not obtain, quantitative measurement is not possible by these methods. A relative measure of energy metabolism was obtained by expressing the activity of each brain region as a percentage of the mean value from right and left occipital cortex in each animal. The results are shown in Table I. There is no significant difference between the right and left sides in any brain region using the paired *t*-test. A one-way analysis of variance showed significant differences ($P < 0.0005$) between the means of the 6 groups in all brain regions on right and left sides, except the frontal cortex. For further analysis data from right and left sides were combined and the Student–Newman–Keul procedure[11] enabled multiple comparisons to be made among the means.

The results of this analysis depicted in Fig. 1 show that in all groups changes in the frontoparietal cortex parallel those in the reference area — the occipital cortex. The effect of amygdaloid kindled convulsions (group 5 compared with group 2) is to cause a relative increase in energy metabolism in the amygdala, hippocampus, septal nuclei and the hypothalamus. In partially kindled animals only 4 brain regions were dissected and the results were intermediate between fully kindled and controls. The changes were bilateral and significantly higher than operated controls (group 2) in the amygdala and hippocampus but not the hypothalamus.

In no area did group 3 differ significantly from group 2 which suggests that the changes observed following kindling in group 5 are the result of the convulsion and not other aspects of kindling. Electroshock gives a different pattern of change from kindling and is accompanied by a relative increase in metabolic activity in all regions studied apart from the parietal cortex (group 6 compared with group 1).

Finally in the hypothalamus and the septal nuclei unoperated controls showed a significantly lower uptake than either of the two control groups with implanted electrodes (group 2 and 3). This finding suggests that electrode implantation to the amygdala may lead to an increased activity in these regions. This effect appears to be bilateral.

The results suggest a functional relationship between the amygdala, hippocampus, septal nuclei and hypothalamus in generalized amygdaloid kindled seizures. This is supported by the finding of less marked and less widespread changes in partial kindling and more widespread changes associated with the generalised seizures induced by electroshock.

The authors wish to thank Dr. J. McQueen and Dr. G. Arbuthnott for advice and encouragement in this project and Dr. B. Meldrum for helpful comments on the manuscript.

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Presynaptic cholinergic modulation of endogenous dopamine and dihydroxyphenylacetic acid release from superfused rat striatal slices

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The influence of the cholinergic system on endogenous striatal dopamine (DA) release and metabolism was studied *in vitro* with the aid of the sensitive electrochemical detection for catecholamines (Felice, Felice & Kissinger, 1978). Striata were dissected out of rat-brain slices (1 mm thick) and cut twice at right angles, at 0·3 mm intervals with a McIlwain tissue chopper. The tissue pieces were separated and preincubated in oxygenated Krebs-bicarbonate buffer (KBB) for 10 min at 37 °C, and then transferred to small chambers (0·2 ml. capacity) and superfused at 37 °C (0·25 ml./min) with oxygenated KBB containing 10^{-4} M-ascorbic acid. After a 15 min washout, the superfusate was collected every 2 min using a fraction collector. Samples were acidified with HCl (final concentration 0·1 mM) containing EDTA (0·1 mM) and the DA/DOPAC separated by high performance liquid chromatography (25 cm–5 μ Hypersil ODS column, mobile phase 0·1 M-KH_2PO_4-citrate buffer containing 30 mg/l. sodium octyl sulphate, 10% methanol and 0·1 mM-EDTA, pH 4·0) and assayed by electrochemical detection (Carbon Paste electrode set at +0·65 V).

Two-minute depolarizations with high K^+ KBB (Na^+ replaced by K^+, 25–50 mM) resulted in DA release which was related to K^+ concentration and was Ca^{2+}-dependent. An increase in DOPAC release followed (by about 2 min) and K^+-induced release of DA and was similarly K^+-concentration and Ca^{2+}-dependent.

Routinely, two treatments with submaximal concentrations (25 mM) of high K^+ KBB (S_1, S_2) were applied. S_2 being initiated 20 min after S_1 was completed. Addition of physostigmine (10^{-8} to 10^{-5} M) to the KBB after S_1 resulted in a dose-dependent increase in S_2 (with a maximal increase of 275% for DA and 320% for DOPAC). Neostigmine (10^{-6} M) had a similar effect which was blocked by atropine (10^{-6} M) but not by gallamine (10^{-6} M) or tubocurarine (10^{-6} M). The increase in K^+ evoked release after acetyl choline (10^{-4} to 10^{-2} M) was potentiated by physostigmine (10^{-8} M) and was also blocked by atropine (10^{-6} M). Atropine (10^{-6} M) alone reduced the release of DOPAC slightly.

These results, which parallel *in vivo* studies (O'Keefe, Sharman & Vogt, 1970; Perez-Cruet, Gessa, Tagliamonte & Tagliamonte, 1971), suggest the presence of cholinergic presynaptic receptors, muscarinic in type, on dopaminergic nerve terminals in the striatum. These receptors appear to mediate the cholinergic stimulation of DA release and turnover.

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RELEASE OF ENDOGENOUS DOPAMINE FROM STRIATAL SLICES 'IN VITRO': COMPARISON OF RELEASE FOLLOWING HIGH K^+ AND ELECTRICAL STIMULATION

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Recent improvements in the methods for detecting catecholamines have allowed the measurement of endogenous transmitter overflow from slices of rat brain superfused 'in vitro' (Bennett et al, 1981; Nahorski & Strupish, 1981; Kapoor, 1981).

The release of dopamine (DA) and its deaminated metabolite 3,4 dihydroxyphenyl-acetic acid (DOPAC) from small slices of neostriatum from rat brain superfused 'in vitro' was estimated after High Performance Liquid Chromatography (5µ.ODS-Hypersil Column, 0.1 M Phosphate - Citrate buffer pH 4.0 with 20mg/l Sodium Octyl Sulphate, 10% Methanol & 0.1mM EDTA) and electrochemical detection on a Carbon paste electrode (+0.65V). During superfusion with Krebs-bicarbonate buffer (KBB) the output of DA and DOPAC was approximately 0.5pmole/mg protein/min and 1.9 pmole/mg protein/min respectively. Superfusion for 2 min with KBB containing 25mM K^+ (K^+ replacing 20mM Na^+) raised these to about 6.5 pmole/mg protein/min (DA) and 3.7 pmole/mg protein/min (DOPAC) (averaged over 6 min from the onset of stimulation). Reducing the Ca^{++} in the superfusate to zero, reduced the output in the presence of high K^+ to control levels.

Electrical stimulation of the slices was performed through silver electrodes at either end of the superfusion chamber. DA and DOPAC levels were increased by stimulation (200 biphasic pulses 2ms wide and 8mA at 2 - 200Hz) but the effects of such stimulation were comparatively small. The overflow of DA increased in the presence of Nomifensine (10^{-6} M). Figure 1 illustrates the frequency dependence of transmitter overflow under these conditions.

In contrast to this marked effect on electrically induced release Nomifensine had no detectable effect on the overflow of DA and DOPAC when high K^+ was the stimulus.

The effects of muscarinic agents on DA overflow which were demonstrated with K^+ stimulation (Kapoor, 1981) can also be seen with electrical stimulation which suggests that the release of DA is still sensitive to some kinds of presynaptic modulation even when increased extracellular K^+ is used as stimulus.

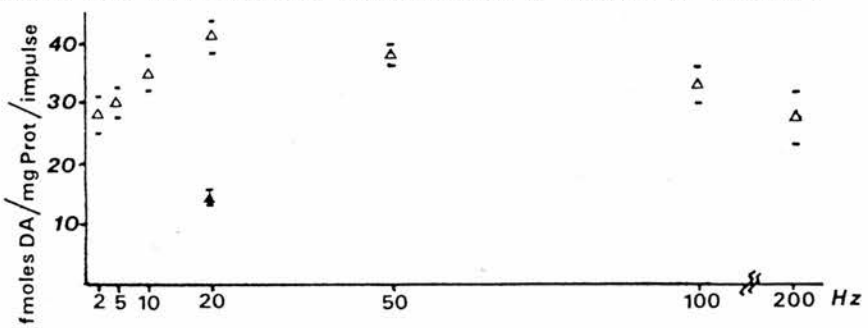

FIGURE 1. Dopamine overflow in the presence (△) & absence (▲) of Nomifensine.

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D.10

CHARACTERISTICS OF ENDOGENOUS DOPAMINE AND DIHYDROXYPHENYLACETIC ACID (DOPAC) RELEASE FROM SUPERFUSED SLICES OF THE RAT STRIATUM

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Although there have been many studies on the release of radioactive Dopamine (DA) from striatal slices, we have taken the opportunity afforded by the electrochemical detection method together with High Performance Liquid Chromatography (HPLC) separation to study the release of endogenous DA (Plotsky et al., 1977).

Striatal slices (0.3 x 0.3 x 1mm) from the head of the Caudate were preincubated in Krebs Bicarbonate Buffer (KBB) containing 10µM ascorbic acid, for 10 min, transferred to small chambers and washed for a further 20 min at a flow rate of 350µl per min. The superfusate was then collected at 2 min intervals using a fraction collector, into tubes containing an internal standard (Dihydroxybenzylamine 0.5ng), HCl and EDTA (final concentration 0.1M and 0.1mM respectively).

The superfusate was directly analysed for DA and DOPAC by HPLC with 5µ-Hypersil ODS column using a 0.1M Phosphate-Citrate buffer, pH 4.0, containing 10% Methanol, 25mg/l Sodium Octyl Sulfate and 0.1mM EDTA, Flow rate was set at 0.8ml/min giving a pressure of approximately 1000p.s.i. Electrochemical Detection was performed with carbon paste, CPO, electrode set at +0.65V, with respect to a Ag/AgCl reference electrode. Under these conditions the retention times for DA and DOPAC were 8 min and 12 min respectively.

Resting release of DA and DOPAC was found to be about 0.5pMol/mg Protein/min and 1.9pMol/mg Protein/min respectively. Superfusion for 2 min with KBB containing 25mM K+ (K+ replacing 20mMNa+)raised these values to about 6.5pMol/mg Protein/min (DA) and 3.7pMol/mg Protein/min (DOPAC) (averaged over 6 min from the onset of stimulation). Release of DA by high K+ KBB was found to be maximal at 35-40mM K+, and completely dependent on the presence of Ca++ in the KBB (Kapoor, 1982).

Electrical stimulation, performed through silver electrodes at either end of the superfusion chamber, was maximal at 8-10mA(2msec biphasic pulses) and at 20Hz (Kapoor and Arbuthnott, 1982). Electrical release of DA was also Ca++ dependent. The release of DA per impulse was found to decrease with increasing number of pulses.

Several differences between the two methods of inducing release have been demonstrated. Dopaminergic uptake inhibitors Nomifensine (10^{-6}M) and LY5953A (10^{-6}M) increased the electrically induced release of DA markedly, they were however without effect on the high K+ evoked release of DA. Acetylcholine-esterase inhibitors, which revealed a clear muscarinic increase in DA release after high K+ stimulation (Kapoor 1982) have much more complex effects on electrically induced release.

(LY5953A was kindly supplied by E.Lilley & Co.)

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COMPARISON OF DOPAMINE OVERFLOW FROM STIMULATED SLICES OF THE NEOSTRIATUM AND MEDIAN EMINENCE 'IN VITRO'

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Because of the greater clinical accessibility of the hormones which they control, the dopamine containing neurones of the median eminence are often used as a model system through which to examine the functional state of central dopaminergic systems in general. The purpose of the present experiments was to compare, in as direct a manner as possible, the release of transmitter from the dopamine containing terminals of the neostriatum with that from the median eminence terminals.

Both areas were dissected from cooled rat brain and preincubated for 20 min. in Krebs bicarbonate buffer at 37°C. Tissue pieces from both areas were then transferred to small perspex chambers and superfused with the same Krebs buffer. The release of dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) was assayed in the superfusate collected at two minute intervals from the chambers. The samples were analysed by high performance liquid chromatography on a 5μ ODS-Hypersil column (0.1M phosphate-citrate buffer, pH 4.0, with 20mg/l Sodium Octyl Sulphate, 10% Methanol and 0.1mM EDTA), and the dopamine and DOPAC in the samples measured by electrochemical detection on a carbon paste electrode (+0.65 V). The retention times of dopamine and DOPAC were 7 min. and 12 min. respectively. (Kapoor and Arbuthnott, 1982).

Median eminence samples from four rats weigh only about 1.5mg but the dopamine output in response to electrical stimulation at 20Hz (2msec biphasic pulses), for 30sec at 10mAmps, is easily measureable and is about 17fmoles/mg protein/impulse, which compares well with the 7fmoles/mg protein/impulse from the striatum.

Several differences in the characteristics of release are worthy of note. The most striking is the very small amount of DOPAC coming from the median eminence slices. Whereas DOPAC is a major component of the output from striatal slices, the resting output of DOPAC from the eminence slices is very small, usually below the detection limits of the assay even during stimulation. Umezu & Moore (1979) also report low levels of DOPAC in tissue from the median eminence. The smaller volume of tissue involved is reflected in the fact that pooled tissue from several rats is required for the median eminence samples, while ample dopamine is released from single slices of neostriatum from one side of one rat brain in the experiments on that area. The effect of the uptake inhibitor, Nomifensine, is clear on the stimulated release from the striatum where at 10^{-6}M it causes a three fold increase of dopamine overflow. However Nomifensine (10^{-6}M) has a very much smaller effect on the median eminence slices, increasing the overflow of dopamine by only about 50%. These results may be explained by the suggestion that the median eminence neurones lack a high affinity dopamine uptake system (Demarest and Moore, 1979), although it has been shown (Sarkar et al., 1981) that Nomifensine does block the uptake of ^{3}H dopamine into similar slices of the median eminence.

These experiments suggest that the control of dopamine release and metabolism in the median eminence is different from that in the striatum and thus counsel caution in the use of neuroendocrine markers to monitor the functional activity of dopamine in areas of the brain other than the median eminence.

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THE RELEASE OF DOPAMINE (DA) INTO HYPOPHYSIAL PORTAL BLOOD IN THE MALE HYPERPROLACTINAEMIC RAT. A. Brar*, V. Kapoor*, A. McNeilly*† G. Fink (SPON: D.W. Lincoln). MRC Brain Metabolism Unit, Department of Pharmacology, 1 George Square, Edinburgh, U.K., and †MRC Reproductive Biology Unit, 37 Chalmers Street, Edinburgh, U.K.

Studies in vitro and the measurement of DA in the hypothalamus have suggested that prolactin (PRL) may increase DA release into hypophysial portal vessel blood and thereby invoke a negative feedback system. We have investigated this possibility by measuring DA in hypophysial portal vessel blood in control adult male rats and in male rats made hyperprolactinaemic by transplanting two pituitary glands under the kidney capsule. Hypophysial portal vessel blood was collected (Fink, G. and Jamieson, M.G., J. Endocrinol. 68: 71, 1976) for 30 min before, during and after the application of an electrical stimulus (30 sec trains of biphasic square wave pulses, 60 Hz, 1mA peak to peak and 1 mA duration) to the median eminence. The portal blood was collected into Trasylol (20,000 KIU/ml) and EDTA (5.4 mM l^{-1}) at 4°C. The concentrations of DA and DOPAC were measured in plasma extracts using HPLC with electrochemical detection (Plotsky, M. et al, Endocrinology 102: 1887, 1978).

Concentration of DA and DOPAC in hypophysial portal plasma (ng/ml, mean ± S.E.M.)

	Pre-stimulation	Stimulation	Post-stimulation
CONTROLS (n=6)			
DA	3.62 ± 0.55	2.66 ± 0.98	2.20 ± 0.07
DOPAC	7.20 ± 0.24	4.60 ± 0.28	7.00 ± 1.98
HYPER-PRL (n=5)			
DA	4.48 ± 0.34	4.56 ± 0.39	-
DOPAC	9.47 ± 1.32	12.20 ± 1.10	-

The table shows that DA and DOPAC levels were slightly higher in the hyperprolactinaemic group than in the control group, although the differences were only significant in the sample collected during stimulation. These results suggest that long-term elevations in plasma PRL (approx. 120 ng NIH-RP-1/ml) elevate the turnover rate of the tuberoinfundibular DA system without significantly affecting the DA concentration in portal blood. Furthermore, despite a 47% depletion of DA concentration in the median eminence (ME) produced by long-term hyperprolactinaemia (Simpkins, J.W. et al, Life Sci. 30: 1249, 1982), electrical stimulation of the ME can augment the PRL-induced elevation of DA turnover while keeping DA concentration constant.

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